THE BIOCHEMICAL JOURNAL

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Respiration and Phosphorylation in Preparations from Mammalian Brain

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Glucose constitutes the main substrate supporting the functional activity of the brain. The main end products from glucose in the brain *in vivo* are lactic acid and carbon dioxide, but the proportions in which these are formed vary greatly with the activity of the brain (for a recent assessment see McIlwain, 1950*b*). These are also the main end products from the metabolism of glucose by slices of mammalian brain *in vitro*. We accordingly decided to study the action of substances which are known to alter the balance of carbohydrate metabolism in brain *in vivo* and *in vitro*. These include narcotics, convulsants, and certain compounds which increase aerobic lactic acid formation. As oxidative phosphorylation constitutes, in brain as elsewhere, a link between functional activity and the metabolism which supports such activity, we have studied the action of the compounds on this process.

The systems studied included that of Ochoa (1941), in which the phosphate esterified during oxidation is transferred to glucose and the phosphorylation followed by determining the loss of inorganic phosphate. In addition, a system has been devised in which labile phosphoric esters themselves can accumulate and be determined together with determination of loss of inorganic phosphate. This does not involve secondary reactions with glucose, and thus has some advantages in studying the actions of inhibitors on the phosphorylation itself.

EXPERIMENTAL

Reagents. Cytochrome *c* was prepared from horse heart according to Keilin & Hartree (1937). It was finally dialysed against water, standardized and preserved in a refrigerator as a solution without $CHCl_3$, as described by Potter (1945*b*).

Potassium pyruvate was prepared in the light of the observations of Lipschitz, Potter & Elvehjem (1938) and Ochoa (1941) on the care necessary to avoid inhibitory substances. Pyruvic acid, purchased as a fine chemical, was distilled twice *in vacuo* and the fraction boiling at 55–60° (10 mm.) diluted to 5 M and kept in a refrigerator. For use it was diluted to 1 M, cooled in ice water, and brought to pH 7·4 with cold KOH. Purity of the pyruvate was checked by use of carboxylase. For determination of pyruvate in reaction mixtures, a method (Lardy, 1945), based on Case's (1932) dinitrophenylhydrazine procedure, was used.

Adenosinetriphosphate, purchased as Ba salt, was suspended in water and dissolved by adding the minimum quantity of M-HCl, keeping cold throughout. The theoretical quantity of K_2SO_4 was then added, the precipitated $BaSO_4$ centrifuged off, and the supernatant neutralized with KOH, still keeping cold. An 0·015 M solution was stable for a week or more when kept at 0°. With the specimen of triphosphate used, the final solution contained inorganic phosphate to the extent of about 8% of its labile phosphate. Adenosine-5-phosphoric acid, purchased, was dissolved in KOH to give an 0·015 M solution.

Homogenates. Glass homogenizers of Potter & Elvehjem's type were used, made from stout tubes about 2·5 cm. internal diameter and driven directly by a $\frac{1}{4}$ h.p. motor (max. speed, 2000 rev./min.). The tube with 3 ml. of the suspending medium was weighed and cooled in ice water prior to use. A guinea pig was killed by decapitation, sometimes preceded by a blow on the neck, the cerebral hemispheres placed in the homogenizer tube and the whole weighed. Homogenizing was done by passing the pestle four times through the contents of the tube with maximum hand pressure, and occupied 4 sec. The tube was immediately returned to ice, and saline added to the contents to make a 1:5 suspension. The suspension was made uniform by using the pestle as a plunger without the motor running, and passed through a wisp of glass wool in a funnel to a test tube, also cooled in ice. This suspension was ready to pipette into experimental vessels 5 min. after the death of the animal, and its mean dry weight after subtracting the weight due to saline was 35 mg./ml. Few, if any, whole cells remained in the homogenate. The cerebral hemispheres weighed approx. 2 g., yielding approx. 9 ml. of filtered homogenate.

Dialysis of homogenates. This must be rapid, as constituents of the phosphorylating system are labile; dialysis through cellophan tubing, obtainable commercially, has been found too slow. A suitable batch of collodion (for semi-permeable membranes: British Drug Houses Ltd.), already in solution, was added to an equal volume of a mixture of 11 vol. ethanol and 50 vol. diethyl ether, mixed, and the bottle left for some days to clear from bubbles. Tubes (12·7 × 1·6 cm.) were filled with the solution and emptied in about 30 sec. while rotating them to coat the walls uniformly; when only a few drops remained they were clamped upside down and left for 0·5 hr. at room temperature to drain and dry. A second coat was then applied in the same way. After the second drying (0·5 hr.) the membrane was moistened, removed from the tube and kept in water until needed. For dialysing homogenates, several litres of 0·05 M-phosphate buffer (from KH_2PO_4 and NaOH, pH 7·4) were kept overnight in a refrigerator. 2 l. were put into a 4 l. beaker, in a refrigerator, the homogenate put into a collodion tube and suspended in

the beaker, and the contents stirred with a motor, sufficiently powerfully to shake the sac well. Two sacs could be run together in 4 l. of phosphate.

Phosphorylation of glucose with dialysed homogenates. The reaction mixtures always contained glucose, fluoride, adenosinetriphosphate and potassium and magnesium salts, with sometimes additional phosphate and fumarate. Choice of the reactants and the concentrations used initially were based on the work of Banga, Ochoa & Peters (1939), and Ochoa (1941), who studied brain preparations, and also of Potter (1945*a*), Green, Loomis & Auerback (1948) and Cross, Taggart, Covo & Green (1949) who investigated primarily kidney preparations. The experiments described in a later section were carried out to choose concentrations optimal for the present working conditions. These gave the following as the most generally adopted procedure.

For an experiment with some eight reaction mixtures, the following were mixed: M-glucose, 0·3 ml.; 0·015 M-potassium adenosinetriphosphate, 0·9 ml.; M-NaF, 0·3 ml.; 0·3 M-sodium fumarate, 0·1 ml.; 0·25 M-potassium pyruvate, 0·9 ml.; water or other addition, 0·2 ml. Warburg vessels (conical, with one side arm and a centre well; of about 15 ml.) were fixed in an ice tray, NaOH pipetted into their centre wells and 0·3 ml. 50% (w/v) trichloroacetic acid pipetted into their side arms. Water, or a solution whose effect was being studied (0·1 ml.), was pipetted into their main vessels, followed by the above mixture (0·3 ml.), the homogenate (1 ml.) and 0·16 M-$MgCl_2$ (0·1 ml.). Papers were immediately put into the NaOH, and the vessels attached to the manometers and placed in the thermostat (37°). After 2·5 min. the joints were rubbed home and shaking commenced. After another 2·5 min. the manometers of experimental vessels were levelled and closed, while those of the controls were removed from the thermostat, their trichloroacetic acid mixed, the vessels detached and returned to the ice tray. Manometric readings for the remaining vessels were taken at intervals of 3 min. usually for 9 or 15 min., after which the reactions were stopped as described before. The series of vessels were put into and taken out of the bath at regular intervals of 15 or 30 sec. To all vessels, now in ice, 1·5 ml. water was added, the solutions each thoroughly mixed with a Pasteur pipette and transferred through coarse fluted papers to test tubes in ice. From these, portions were taken for analysis.

Phosphorylation with more dilute homogenates. Homogenates were prepared in some cases as described above, and in others by homogenizing for about 0·5–1 min. in a homogenizer with a looser pestle and less powerful motor; they were not dialysed. Except where specified otherwise, the cerebral hemispheres were added to 4 vol. of a solution containing 0·6% potassium chloride and 0·5% sodium fluoride. The mixture was used immediately after homogenizing, pipetting 0·6 ml. to the side arms of already prepared Warburg vessels; it was at pH 7 without any adjustment.

The vessels contained 2·4 ml. of reaction solution in their main compartments and KOH and paper in their centre wells. The reaction mixture most generally adopted contained in the final reaction mixture of 3 ml.: $8·3 \times 10^{-2}$ M-glycylglycine buffer, pH 7·4; $1·7 \times 10^{-3}$ M-phosphate buffer from KH_2PO_4 and NaOH, pH 7·4; $1·3 \times 10^{-2}$ M-KCl (from homogenate); $2·1 \times 10^{-2}$ M-NaF (from homogenate); $1·7 \times 10^{-2}$ M-$MgCl_2$; $3·3 \times 10^{-3}$ M-sodium fumarate; $3·3 \times 10^{-2}$ M-potassium pyruvate; $3·7 \times 10^{-5}$ M-cytochrome *c*; 10^{-3} M-potassium adenosinetriphosphate; 10^{-3} M-potassium adenosine-5-phosphate. The constituents were prepared so that the majority could be mixed together for a group of six to ten vessels, and added as 2 ml. of solution. The homogenate and added substances whose effects on phosphorylation were being examined were added separately.

The course of the manometric experiment was similar to that described above, the reaction again being stopped by adding trichloroacetic acid, either from a second side arm or by pipette after detaching the vessel.

Determination of phosphates. Inorganic phosphate was determined on portions of trichloroacetic filtrates, according to Lowry & Lopez (1946). The molybdate reagent was kept in a waxed bottle at 2°, and the ascorbic acid used for reduction was prepared immediately before use. The molybdenum blue was read in a Beckman quartz spectrophotometer at 700 mμ. in a volume of 3 ml. Standards of about 1 μg. P/ml. were used for comparison. With this instrument, only occasional and small interference was observed with any of the coloured heterocyclic compounds whose action on phosphate distribution was being studied. Corrections were applied where necessary by taking blank readings after addition of the ascorbate but before addition of the molybdate.

The labile phosphates (possibly adenosinetriphosphate, diphosphate or pyrophosphate) were determined as follows. After trichloroacetic precipitation of either slices or homogenates, the cold suspensions were filtered into test tubes standing in ice. The filtrate was divided and inorganic phosphate determined on one portion. Another (1 ml.) was mixed with 1 ml. 2 N-HCl, the tubes placed in a boiling-water bath for 7 min. and then cooled in ice. NaOH (0·4 ml., 5 N) and sodium acetate (0·1 M, to 5 ml.) were added, and inorganic phosphate redetermined; the increase is referred to as labile (7 min.) phosphate. P/O ratios are calculated in atoms.

RESULTS

Dialysed homogenates

Respiration. Conditions were based initially on those of Banga *et al.* (1939) and Ochoa (1941). Homogenates were studied initially with the additions of fumarate, glucose, magnesium salts, adenosinetriphosphate and fluoride which are indicated above, and examined in the presence and absence of pyruvate. Respiration was high and only slightly affected by added pyruvate; dialysis was carried out in thin collodion sacs for 75 min. This gave a preparation whose respiration (see Table 1, Exps. 1–3) was dependent on added pyruvate, fumarate and magnesium and was almost linear during the short period required. Experiments with different concentrations of the reagents led to the choice of those indicated above, which are close to those of other authors.

Table 1. *Phosphorylation in dialysed homogenates*

(Reaction mixtures of 1·5 ml. were prepared as described in the experimental part. Final concentrations of reactants were: glucose, 0·022 M; adenosinetriphosphate, 0·001 M; NaF, 0·022 M; sodium fumarate, 0·002 M; potassium pyruvate, 0·017 M; $MgCl_2$, 0·01 M and potassium and sodium phosphates (from the homogenate) approx. 0·02 M.)

Exp. no.	Changes in reaction mixture	Oxygen uptake (μmol./vessel)	Loss of inorganic phosphate (μmol./vessel)	P/O ratio (atom/atom)
1	None	4·15	18·2	2·2
	Anaerobic	0	1·0	—
2	None	2·50	12·5	2·45
	No pyruvate or fumarate	0·65	0·8	0·61
	No Mg	1·9	1·7	0·4
3	None	4·35	15·5	1·8
	Fluoride to 0·03 M	4·16	15·0	1·8
	Fluoride to 0·036 M	3·5	13·1	1·87
4	No fluoride	5·0	0	—
5	None	2·54	11·0	2·25
	Ethyl red 2×10^{-7} M	2·15	6·3	1·46
	Ethyl red $6·7 \times 10^{-7}$ M	1·95	4·25	1·12
	Ethyl red 2×10^{-6} M	1·70	3·0	0·91
	Ethyl red $6·7 \times 10^{-5}$ M	1·06	1·0	0·47
6	None	3·58	18·5	2·6
	2:4-Dinitrophenol 5×10^{-5} M	3·70	10	1·3
	2:4-Dinitrophenol $1·66 \times 10^{-4}$ M	2·64	0·4	0·1
7	None	3·38	14·7	2·17
	Phenosafranine $6·67 \times 10^{-5}$ M	2·35	9·8	2·08
	Phenosafranine 10^{-4} M	1·55	1·0	0·3
	Phenosafranine $1·33 \times 10^{-4}$ M	1·0	0·3	0·15
8	None	3·53, 3·30	19·2, 17·9	2·70, 2·7
	Chloral 1·5 mg./ml.	1·77, 1·79	15·6, 13·95	4·4, 3·9
9	None	2·74, 2·58	14·5, 14·5	2·66, 2·82
	Chloral 1·5 mg./ml.	1·20, 1·21	9·3, 9·3	3·88, 3·84
10	None	2·66	15·1	2·84
	Chloral 0·75 mg./ml.	1·65	10·9	3·3
11	None	3·67	6·7	0·91
	Chloral 0·75	3·36	7·8	1·16
	Chloral 1·5	2·43	5·9	1·21
	Chloral 2·25	1·68	4·2	1·25
12	Fumarate $1·5 \times 10^{-4}$ M	1·53	4·8	1·6
	Fumarate $1·5 \times 10^{-4}$ M; phenosafranine 4×10^{-5} M	1·43	2·8	0·98
	Fumarate $1·5 \times 10^{-4}$ M; phenosafranine 6×10^{-5} M	1·3	0·7	0·27
	Fumarate $1·5 \times 10^{-4}$ M; phenosafranine 8×10^{-5} M	1·3	0·1	0·04
	Fumarate $1·5 \times 10^{-4}$ M; phenosafranine 10^{-4} M	1·06	1·2	0·5

The addition of fluorides during these experiments was necessary to prevent loss of the phosphates esterified, as active adenosinetriphosphatases exist in brain (Meyerhof, 1947; Utter, Reiner & Wood, 1945; Meyerhof & Wilson, 1948). Exps. 3 and 4 of Table 1 illustrate its action. Variations in fluoride concentrations between 0·02 and 0·03 M had little effect on respiration, but 0·036 M depressed it by about 25%. At the concentration of 0·022 M chosen finally, the respiratory quotient was determined during the oxidation of pyruvate, by the method of Warburg & Yabuso (1924). For the oxidation occurring during the first 9 min. an approximate value of 1·4 was found, and during the first 27 min., one of 1·22. Complete oxidation of pyruvate requires a value of 1·2.

The rate of respiration during different experiments with the full reaction mixture has been between 0·48 and 0·74 μmol./mg. dry wt./hr., or 81–127 μmol./g. corresponding fresh wt./hr. An example is given in Fig. 1. The 1 ml. of dialysed homogenate of a typical experiment contained material from 200 mg. fresh weight of brain, which was found to have 34–36 mg. dry wt. With the lower concentration of fumarate used in some experiments (Table 1, Exp. 12), respiration was at the rate of 0·18 μmol./mg. dry wt./hr., or 31 μmol./g. fresh wt./hr.

Phosphorylation. Rapid disappearance of inorganic phosphates was found aerobically, but not with the same reaction mixture in the absence of oxygen (Table 1, Exp. 1). In the absence of fluoride no inorganic phosphate was lost. During experi-

ments lasting 9 or 15 min. the ratio of inorganic phosphate lost to oxygen absorbed (ratio atoms P/atoms O) varied in different experiments from 1·6 to 3·0, with 2·4–2·8 as the most frequent range. The course of phosphorylation was not, however, found to be entirely uniform, and is illustrated in Fig. 1. The initial lag in phosphorylation is understandable, as zero time in the figure is the time at which the cold flasks are placed in the thermostat. Rapid phosphorylation is normally beginning at the time at which manometric observations of oxygen uptake are commenced, and phosphorylation is again slowing at the end of the 9 min. experimental period. During a brief period P/O ratios may be higher than average for the whole period, which is that quoted in Table 1.

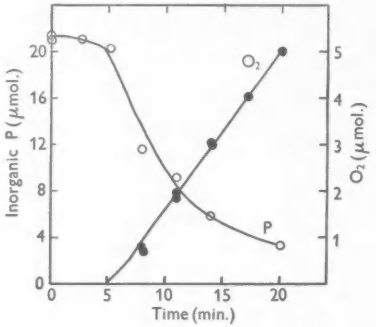

Fig. 1 Course of respiration and phosphorylation in dialysed homogenates. The reaction mixture was that described as normal in the experimental part, and was contained in eight vessels in which the reaction was stopped at the different times indicated. Dry weight of homogenate used/vessel, 34 mg. P/O ratio between 5 and 14 min.: 2·55.

Effect of added substances on respiration and phosphorylation. The effects of several substances on respiration, on loss of inorganic phosphate, and on the P/O ratio are quoted in Table 1. The substances include phenosafranine and ethyl red, which increase aerobic glycolysis in brain slices (Dickens, 1936; McIlwain & Grinyer, 1950), and 2:4-dinitrophenol (Loomis & Lipmann, 1948) which, as well as phenosafranine (Judah & Williams-Ashman, 1949), has been reported to inhibit the phosphorylation associated with oxidation in preparations from other tissues. Each of these substances lowered the P/O ratio in the present experiments. Chloral was examined also; this inhibited respiration and phosphorylation to about the same degree, so that the P/O ratio changed little or was slightly increased.

The results suggested that substances of the type of 2:4-dinitrophenol and phenosafranine were indeed inhibitors of aerobic phosphorylation. There were, however, certain sunatisfactory features about the present findings, the main one being that the lowering of phosphorylation was usually associated with some inhibition of respiration. The different substances studied differed in this respect; the difficulty of discriminating between effects on respiration and phosphorylation was greater with phenosafranine than with 2:4-dinitrophenol. The inhibition of respiration was a phenomenon not seen in slices when these contained comparable concentrations of phenosafranine (McIlwain & Grinyer, 1950), and it varied in different reaction mixtures with homogenates. Thus Table 1 shows that the inhibition of respiration was less in homogenates which had a low concentration of fumarate; but here respiration was slow and the P/O ratio low.

It was also necessary in assessing experiments with inhibitors to consider the course of phosphorylation and respiration, and this is shown in Fig. 1. During the period of 5 min. equilibration before manometric readings were taken, phosphate changed relatively little; after this, rapid change took place until three-quarters of the inorganic phosphate was lost. Respiration was almost linear during the whole of the period of observation. In a normal experiment the period during which changes were observed was in the 9–12 min. after beginning respiration unless respiration was observed to be slow (e.g. with low fumarate concentrations), in which case experiments were continued for 15–20 min. During this time the changes in phosphate were approximately but not exactly linear. It is presumably such departure from linearity which permits the apparent increase in P/O ratio observed with chloral. It would have been desirable to observe the whole of the changes in both oxygen and phosphate by mixing the homogenate with the reaction fluid after the period of equilibration, but the phosphorylating systems in such dialysed homogenates are very labile to even a brief warming at 37° in the absence of substrates. These and other considerations led to the use of non-dialysed homogenates described below.

Non-dialysed, more dilute homogenates

A system more suited for examining the action of inhibitors on phosphorylation has been devised as follows: (i) the reactions involved in phosphorylation have been decreased in number by following the accumulation of labile phosphates themselves, rather than the result of the further transfer to glucose; (ii) the homogenate has not been dialysed, so that its phosphorylating systems remain more stable; (iii) the reaction conditions have been brought closer to those found by Elliott & Libet (1942), Elliott, Scott & Libet (1942) and Larner, Jandorf & Summerson (1949) to be optimal for respiration of brain homogenates. The addition of fluoride to minimize loss of adenosinetriphosphate remains necessary.

Respiration. Dilution of the dialysed homogenate led to a rapid fall in its respiration and phosphorylation, but with the present experimental arrangement, without dialysis, respiration remained high at concentrations of brain of some 6–8 mg. dry wt./ml. This enabled inorganic phosphate and adenosinetriphosphate to be used at concentrations approaching those normal to the tissue, and 1 to 2×10^{-3}M concentrations were chosen. Adenosine-5-phosphate was included as phosphate acceptor, also at 10^{-3}M. The low phosphate concentrations made necessary the use of an independent buffer and glycylglycine was chosen after finding veronal to be inhibitory.

Although labile phosphates are synthesized in the present reaction mixture, it was found necessary to add adenosinetriphosphate, as such, for optimal respiration and phosphorylation. This is shown in Fig. 2, together with the effect on respiration of omitting other constituents of the reaction mixture. Magnesium salts were seen to be necessary for optimal respiration and a similar experiment showed this to be the case also with cytochrome *c*. Respiration is, however, nearly independent of added inorganic phosphate and phosphate acceptors, some of which are already contained in the homogenate. Although cozymase and nicotinamide were found necessary by Larner *et al.* (1949) for optimal respiration in certain brain homogenates, we did not include them in the normal reaction mixture as we specifically wished to study the effects of such substances on phosphorylation. When added in the concentrations suggested by Larner *et al.* (1949) the compounds had little effect on respiration; our preparations presumably corresponded to the 'coarse homogenates' of these authors. The present preparations were found to retain some 25% of the cozymase of the whole tissue, for at least 30 min. (Gore, Ibbott & McIlwain, 1950). Air was used in the gas space during most of the experiments. Oxygen appeared to have some very slight advantage when the manometric apparatus was stopped frequently for readings. It was never found detrimental to respiration during the present experiments, which usually lasted only 9 or 15 min.; Dickens's (1946) observations, on oxygen poisoning in brain suspensions (see Larner *et al.* 1949) concerned much longer experimental periods.

Respiration in the present system without added substrates was higher than with the dialysed homogenates (Fig. 2). The present system is thus less suited for following the fate of the added substrate. Without fumarate, respiration fell to about two-thirds of its rate and phosphorylation fell still further. Doubling or halving the normal concentration of pyruvate led to little change. We have not determined accurately the ratio of changes in pyruvate and oxygen, as the pyruvate was present in the normal mixture in large excess and a relatively small change took place in it. On reducing its quantity to one-fifth, the rate of respiration fell, but some measure of the removal of pyruvate could be obtained. During 60 min. this was found to be about half the molar quantity of the additional oxygen absorbed. (Pyruvate lost: 1·4 of 10 μmol. Oxygen

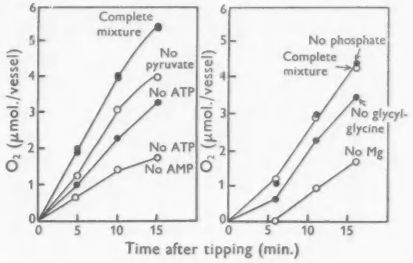

Fig. 2. Effect of different reagents on the respiration of dilute homogenates. Full medium: that described in experimental section, p. 2. ATP, adenosinetriphosphate; AMP, adenosinemonophosphate.

uptake in the presence of pyruvate, 4·73 μmol.; in absence, 1·95.) Although caution is needed in interpreting the change in oxygen uptake, there is some approximation to complete oxidation of pyruvate in the present reactions, and no considerable diversion of it to other reactions.

Phosphorylation. In the absence of fluoride, labile phosphate is lost from the reaction mixture and inorganic phosphate increases. Fig. 3 shows this, and also the actions of a range of fluoride concentrations on respiration and phosphorylation. Inhibition of respiration was noticeable even below 10^{-2}M (for comparable inhibitions in kidney homogenates see Potter, 1945*a*). In spite of this, the yield of labile phosphate as measured by the P/O ratio increased until about 2×10^{-2}M-fluoride was reached; it then fell. Concentrations between 1·6 and $2{\cdot}7 \times 10^{-2}$M were found optimal. On the basis of such results it was decided to incorporate in the phosphorylating system $2{\cdot}1 \times 10^{-2}$M-sodium fluoride. Ochoa (1943) employed $2{\cdot}5 \times 10^{-2}$M-fluoride with a muscle preparation and found comparable inhibition of respiration and of the breakdown of labile phosphates. In the present experiments, fluoride was found to have a greater effect in maintaining labile phosphates when it was present in the initial homogenizing fluid. Its action under these conditions is shown in Table 2. The reaction mixture here lacked only the pyruvate and fumarate of the normal system. Adenosinetriphosphate was added to one pair of vessels to give about 10^{-3}M (as in the reaction mixture), and to another pair $2{\cdot}4 \times 10^{-3}$M, which is somewhat greater than the usual final concentration after phosphorylation. Hydrolysis of labile phosphate is seen to be

fairly well controlled, though some loss occurs with the higher concentrations of adenosinetriphosphate. One result of the incomplete control of phosphatases was that certain potent inhibitors of phosphorylation could lead not only to lack of increase in labile phosphate, but to decrease in its quantity in certain reaction mixtures (Table 3).

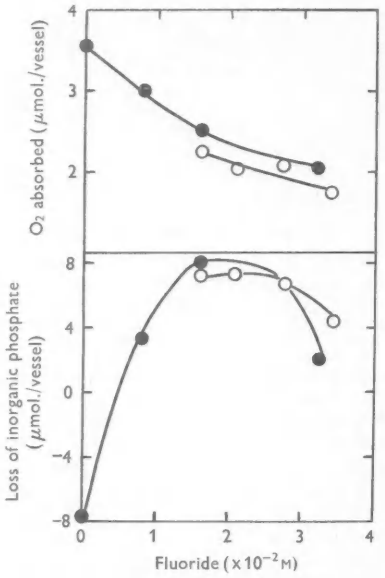

Fig. 3. Respiration and phosphorylation in dilute homogenates containing different concentrations of fluoride. Reaction period, 12 min.; 7 mg. dry weight homogenate/ml. Two experiments, the results being indicated by ● and ○ respectively.

We have not determined the nature of the labile phosphoric ester which accumulates, beyond observing that the whole of the inorganic phosphate which disappears from the reaction mixture is recovered after 7 min. at 100° with N-HCl. It could therefore be the labile phosphate of the adenosine polyphosphates, or inorganic pyrophosphate (see Cross *et al.* 1949). Its quantity was decreased when adenosinemonophosphate was omitted from the reaction mixture. On the other hand, the inclusion of creatine (0·016 M) was found to be without effect on the loss of inorganic phosphate, or on the formation of labile phosphate, in a variety of reaction mixtures.

The phosphorus/oxygen ratio. These findings gave the basis for the reaction mixture described in the experimental part. A series of results with it are quoted in Table 3. This shows that in the 15 min. of a typical experiment, more than half of the inorganic phosphate became esterified and the concentration of labile phosphate was nearly doubled. Substances of the type under investigation could inhibit these changes or, in high concentrations, reverse them. Nevertheless, the inorganic phosphate lost was always accounted for completely, within experimental error, by the 7 min. phosphate formed. The

Table 2. *Control of adenosinetriphosphatase by homogenizing in fluoride*

(Vessels contained glycylglycine, pH 7·4, $8·3 \times 10^{-2}$ M; KCl, $1·3 \times 10^{-2}$ M; cytochrome *c*, $3·7 \times 10^{-5}$ M; sodium fumarate, $3·3 \times 10^{-3}$ M; $MgCl_2$, $1·7 \times 10^{-2}$ M, and homogenate containing 18·6 mg. dry wt. brain. Initial inorganic P was found to be 84 μg. and labile P, 70 or 146 μg./ml. according to the quantity added.)

Adenosine-triphosphate added (10^{-3} M)	Fluoride (10^{-2} M)	Change (μg./ml.) during 15 min. at 37°	
		Inorganic P	Labile P
1·1	0	+61	−57
2·4	0	+109	−105
1·1	2·1	+2	+2
2·4	2·1	+19	−15

ratio between the changes in phosphate and in oxygen, quoted in the last column of Table 3, is comparable to ratios reported by many investigators. It is lower than that obtainable under conditions chosen as optimal for phosphate removal, such as those of the dialysed homogenate described above. The lower ratio in the present experiments appears likely to result from the increased difficulty of controlling adenosinetriphosphatases, caused by the greater concentration of adenosinetriphosphate in the present reaction mixture.

The course of changes in both phosphates and oxygen have been followed and found to be nearly linear during the 10 or 15 min. during which phosphorylation experiments were normally run; both processes had fallen to about three-quarters of their initial rates, at 15 min.

Actions of added substances on phosphorylation by diluted homogenates

2:4-*Dinitrophenol and azide.* The effect of the dinitrophenol in decreasing phosphorylation, noted above, was fully confirmed with the present homogenate. Low concentrations of it also consistently caused a slight increase in respiration associated with the first lowering of phosphorylation. Concentrations of about 10^{-4} M had a nearly maximum effect in lowering phosphorylation, and at this point respiration was little if at all affected (Fig. 4). It is especially notable that concentrations, even ten times those at which lowering of the P/O ratio was evident, did not lower respiration below the control value (Table 3), though still higher concentrations could do so. This latter effect may have been

Table 3. *Phosphorylation with more dilute homogenates: effects of added substances*

(Vessels contained the reaction mixture of Table 2 with K pyruvate, $3{\cdot}3 \times 10^{-2}$ M and NaF, $2{\cdot}1 \times 10^{-2}$ M, and were incubated for 15 min. Respiration was close to linear, with a small initial lag. Vessels were removed and their contents precipitated after 15 min. at 37°. Initial inorganic P, 216 μg./vessel or about 60 μmol./g. brain; labile P, 168 μg./vessel or about 145 μmol./g. brain.)

Exp. no.	Changes in reaction mixture (M concns. of added substances in parentheses)	Oxygen uptake (μmol./g. fresh wt.)	Change in phosphate (μmol./g. fresh wt.)	P/O ratio (atom/atom)
1	No pyruvate	23·4	40·4	0·86
	None	26·3	47·5	0·90
	2:4-Dinitrophenol (10^{-6})	25·0	43·0	0·86
	2:4-Dinitrophenol (10^{-5})	30·8	33·6	0·55
	2:4-Dinitrophenol (10^{-4})	24·3	−15	−0·31
	Sodium azide (10^{-3})	23·3	−8·5	−0·18
	Sodium azide (10^{-2})	17·6	−4·5	−0·13
2	None	16·3	22	0·68
	Synthalin B (10^{-5})	14·8	4·4	0·14
	Synthalin B ($3{\cdot}3 \times 10^{-5}$)	13·0	−1·3	−0·48
	Synthalin B (10^{-4})	9·5	−3·5	−1·32
3	None	12·1	12·2	0·50
	Nicotine (10^{-3})	12·1	10·4	0·43
	Nicotine ($3{\cdot}3 \times 10^{-3}$)	13·0	11·2	0·43
	Nicotine (10^{-2})	12·9	8·4	0·32
4	None	13·6	42	1·53
	Nicotine ($1{\cdot}7 \times 10^{-2}$)	12·1	23·2	0·96
	Nicotine ($3{\cdot}3 \times 10^{-2}$)	13·8	14·4	0·56
5	None	12·0	25·8	1·08
	Diazine green (10^{-6})	13·4	24·2	0·90
	Diazine green ($3{\cdot}3 \times 10^{-6}$)	13·1	22·4	0·86
	Diazine green (10^{-5})	12·6	15·4	0·61
6	None	12·1	12·2	0·50
	Diazine green (10^{-5})	10·8	1·0	0·05
	Diazine green ($3{\cdot}3 \times 10^{-5}$)	7·8	−0·7	−0·05
	Diazine green (10^{-4})	6·1	−1·2	−1·0
7	None	23·2	47·8	1·03
	Neutral red ($1{\cdot}7 \times 10^{-3}$)	21·5	20·2	0·47
	Neutral red (5×10^{-3})	12·8	7·2	0·28
	Pyocyanine ($3{\cdot}3 \times 10^{-4}$)	26·5	41·6	0·78
	Pyocyanine (10^{-3})	25·4	26·4	0·52
8	None	28·8	38·8	0·67
	Pyocyanine (10^{-3})	27·0	18·8	0·35
	Pyocyanine ($3{\cdot}3 \times 10^{-3}$)	18·5	−3·6	−0·10
	Pyocyanine (10^{-2})	7·4	−11·8	−0·80

secondary to the changes in phosphate, as it was always associated with a loss (and not merely a lack of increase) of labile phosphates in the reaction mixture, and absence of adenosinetriphosphate lowers respiration (Fig. 2). The effect of sodium azide was similar to that of the dinitrophenol (Table 3).

Phenazines, nicotine and synthalin. (For structures of some of these see McIlwain, 1950*a*.) These have certain characteristics in common with the dinitrophenol. All show effects on respiration and phosphorylation, which vary markedly with the concentration of added substance. Phenosafranine, diazine green and pyocyanine were observed to increase respiration at the lower concentrations of their active range (Table 3, Fig. 4). In most cases this was associated with lowered phosphorylation. Higher concentrations of these substances then led to inhibition of respiration with much greater inhibition of phosphorylation. Lowered phosphorylation, accompanied by little if any change in respiration, was also observed to follow the addition of nicotine and neutral red. Lowering was also observed with synthalin B (undecane diamidine), but here it was associated with inhibition of respiration.

The results of several experiments on the action of phenosafranine are shown in Fig. 4 in comparison with those of the dinitrophenol. The similarity is considerable, but the figure illustrates also that the differentiation between inhibition of respiration and phosphorylation is not so great with phenosafranine as with the dinitrophenol. The difference in respiration and phosphorylation, between control and experimental vessels, has been selected in Fig. 4*C* as the best means of expressing the results of a number of experiments. This is because the actual level of phosphorylation itself varies with different

homogenates (Table 3). When so expressed, the results in Fig. 4*C*, which are from four different experiments at different times during the course of 6 months, are seen to be in approximate agreement.

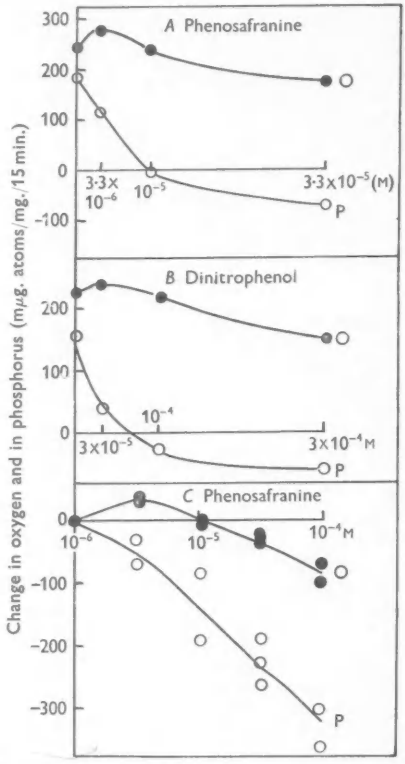

Fig. 4. Effect of different concentrations of phenosafranine and 2:4-dinitrophenol on respiration and phosphorylation in dilute homogenates. Ordinates: *A* and *B*, changes in individual vessels; *C*, differences of experimental from control vessels, in a series of experiments.

DISCUSSION

The balance between lactic acid and carbon dioxide as end products of carbohydrate breakdown in animal tissues has been the subject of many studies. It was early realized that the relation between the two end products was conditioned by the energy made available in their formation, and these relationships have been expressed more recently in terms of energy-rich phosphate derivatives (Johnson, 1941; Lipmann, 1942; Seits & Engel'gardt, 1949).

Brief accounts of the actions on aerobic phosphorylation of agents which affect the course of carbohydrate metabolism have appeared during the past 2 years. Loomis & Lipmann (1948, 1949) found a dinitrophenol to inhibit uptake of inorganic phosphate by a washed kidney suspension oxidizing glutamate; the range of concentration used was 5×10^{-6}–2×10^{-4} M, and respiration was little affected or slightly stimulated. Azide between $1{\cdot}7 \times 10^{-2}$ and $1{\cdot}7 \times 10^{-3}$ M inhibited phosphorylation in a modified system. Judah & Williams-Ashman (1949), also using kidney preparations oxidizing glutamate, found 2:4-dinitrophenol, phenosafranine, and some related compounds to inhibit phosphorylation but not respiration. More recently, Tepley (1949) examined the action of 2:4-dinitrophenol in brain and liver preparations, and Eiler & McEwen (1949) found pentobarbital to inhibit both respiration and phosphorylation in a brain homogenate oxidizing pyruvate. In the present work the actions of several of these substances, and of some others, have been studied under two well defined sets of experimental conditions. This has enabled some comparative observations to be made on the types of effect given by different substances.

The first main differentiation in type of inhibition corresponds to one indicated by Eiler & McEwen (1949) and can be illustrated by the effects of chloral and of 2:4-dinitrophenol. In the concentrations in which it increases lactic acid formation in guinea pig brain slices (Buchel & McIlwain, 1950), chloral inhibits phosphorylation in homogenates of guinea pig brain. The effects both in slices and in homogenates are, however, always associated with inhibition of respiration. In homogenates, this occurs to a degree approximately equal to that of the inhibition of phosphorylation. The ratio of the two changes, or the P/O ratio, is therefore altered relatively little by chloral. These results parallel those reported briefly by Eiler & McEwen (1949) to be characteristic also of another narcotic, phenobarbitone. Hence one may conclude that a respiratory and not a phosphorylating step is primarily involved in the actions of these compounds. Michaelis & Quastel (1941) have already attempted to determine the respiratory step involved in the action of this class of compound. The effects of 2:4-dinitrophenol, when examined under the same conditions as chloral, are characteristically different in that respiration can be unaffected or even increased, while phosphorylation is greatly inhibited. The P/O ratio thus falls. These two types of inhibition of phosphorylation may correspond to the two types of action on aerobic glycolysis found by Warburg (1926) in different substances: an increase in glycolysis with decreased respiration, and an increase in glycolysis without decrease in respiration. Narcotics are cited as examples of the first action.

Differentiation of these two types of action assists in assessing the actions of the other substances examined. Of these, the phenazine derivatives

studied by Dickens (1936), McIlwain (1950*a*) and McIlwain & Grinyer (1950) form the largest group, and of them phenosafranine has been most fully examined. The actions of low concentrations of phenosafranine resemble those of the dinitrophenol, though inhibition of phosphorylation is not so well differentiated from inhibition of respiration. This is, however, a characteristic which varies with the system in which phenosafranine is examined. Differentiation is much better with the diluted than with the dialysed homogenates. The inhibition of respiration by phenosafranine in homogenates is a new phenomenon in the sense that it does not occur in slices which contain comparable concentrations of phenosafranine (McIlwain & Grinyer, 1950). It therefore seems that the action of lower concentrations of phenosafranine are most relevant to its action in slices. Here it is interesting to see that the homogenates retain a characteristic often shown by phenosafranine in slices, namely a stimulation of respiration at the lower concentrations at which its action on phosphorylation and glycolysis first became noticeable. The stimulation is seen also in the action of the dinitrophenol in homogenates.

An immediate conclusion from the findings with 2:4-dinitrophenol and low concentrations of phenosafranine might be that they uncoupled some normal link between phosphorylation and respiration, (Loomis & Lipmann, 1948). This conclusion appears to remain a valid one in spite of the observations of Tepley (1949) on the possible mechanism of the action of dinitrophenol. Before such a conclusion could be accepted with respect to the present system some subsidiary investigations were, however, necessary. The major difficulties in interpretation arose from the presence of phosphatases and the use of fluoride in controlling them. Thus, in the present studies we have considered the following points.

First, an acceleration of phosphatases would give a result equivalent, when examined by the present techniques, to decreased phosphorylation. It was therefore important to examine the effect of, for example, phenosafranine on the adenosinetriphosphatase of brain to see whether change was observed. This has been done (McIlwain, 1950*a*) and negligible effect observed at concentrations equal to, or greater than, those of the present experiments. Secondly, if 2:4-dinitrophenol or phenosafranine caused release of adenosinetriphosphatases from their inhibition by fluoride, the result would again be equivalent to inhibition of phosphorylation. It therefore appeared desirable to attempt an experiment in the absence of fluoride. With the dialysed homogenates this results only in a lack of any change in phosphate, but a graded effect can be seen in the experiments with diluted homogenates in reaction mixtures containing added adenosinetriphosphate. Fig. 5 gives the result of such an experiment. Here 2:4-dinitrophenol and phenosafranine lead to a greater loss of labile phosphate than in their absence; the change in labile phosphate in their absence presumably represents the balance of its synthesis and breakdown. Phenosafranine and the dinitrophenol therefore show the same action in the absence as in the presence of fluoride.

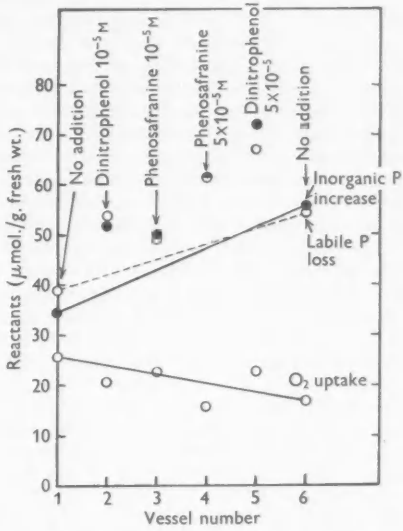

Fig. 5. Change in oxygen and phosphates in the absence of fluoride. After equilibration, brain homogenate was tipped from side arms of vessels to the main compartments, in the order of the vessel numbers quoted. Initial labile phosphate, 79 μmol./g. fresh wt.; duration of respiration, 9 min.

Thirdly, additional problems of interpretation are presented in systems in which the observed P/O ratio (of atoms P esterified/atoms O absorbed) is lower than the maximum. Using glucose or hexose monophosphate as phosphate acceptor, Ochoa (1941) found ratios of up to 2 with brain oxidizing pyruvate, and Cross *et al.* (1949) ratios approaching 2·5 with kidney oxidizing α-ketoglutarate. These values are reached during the present experiments with dialysed homogenates.

In diluted homogenates values are between 0·5 and 1·8, which presumably reflects the greater difficulty in controlling phosphatases during these experiments, when adenosinetriphosphate is present in considerable quantity. Ochoa (1943) noted such variation in phosphorylation in muscle preparations and gave the same explanation. In dialysed homogenates the value of 2·5 atoms/atom may even for a brief period be exceeded in the presence of concentrations of fumarate of the order of 0·02 M; this may

be due to fumarate itself acting as hydrogen acceptor. With lower fumarate both respiration and the P/O ratio are lower and therefore substances which prevent the participation of fumarate would yield a lowered P/O ratio. We have also observed an instance of a secondary lowering of P/O ratio through an action at pyruvate.

It therefore appears that the action of inhibitors in which lowering of P/O ratio is associated with any inhibition of respiration needs very careful assessing before it can be concluded that the primary action of an agent is on a phosphorylating rather than a respiratory step. In considering the actions of the phenazines and nicotine it is therefore especially noteworthy that phosphorylation can be inhibited in the diluted homogenates without any inhibition, or even with an increase, in respiration. To some extent, however, observation of increase in respiration brings its own problems, for it raises the question of whether all the oxygen absorbed is necessarily associated with phosphorylation at the same P/O ratio. If not, a change in overall P/O ratio could result from the action of an agent on some part of the respiration without acting differentially on the phosphorylation associated with it. For this reason we have considered it important to examine the actions of a variety of agents under the same experimental conditions, and observations with chloral form an important control in considering the action of 2:4-dinitrophenol and the phenazines.

It is concluded, therefore, that the lower concentrations of phenosafranine act like the dinitrophenol in inhibiting some step connecting phosphorylation with respiration. There are likely to be many such steps and differential inhibition of these could be of value in disentangling the processes involved. Differentiating characters in the action of dinitrophenol and the phenazines are therefore of interest. The inhibition of respiration by higher concentrations of phenosafranine and its much more potent action in inhibiting cozymase degradation (McIlwain, 1950*a*) may be examples of such characters.

Comparison of the present findings in brain homogenates with the reports available for the actions of similar compounds in preparations from mammalian kidney, show some similarities and some differences. Respiration in the present systems appears more susceptible to 2:4-dinitrophenol, but less so to azide. Thus Loomis & Lipmann (1949) found it necessary to use ferricyanide as hydrogen acceptor in order to dissociate the effect of azide on respiration of kidney homogenates, from its effect on phosphorylation. With brain differential inhibition was seen with oxygen as acceptor (Table 3). Our system contained added cytochrome *c*.

In the case of the phenazines and nicotine in our studies, it is possible to make some quantitative comparisons between their actions on phosphorylation in homogenates and their actions on aerobic lactic acid formation in slices. This is because the concentrations of the different substances in slices have been determined and correlated with their actions on glycolysis (McIlwain & Grinyer, 1950). Pyocyanine is excluded from the comparison because it is chemically changed in the slices, where it exists largely in reduced forms. The comparison is shown in Table 4. The actions on phosphorylation were derived from data such as that of Table 3, plotted as shown in Fig. 4. Two arbitrary degrees of action on phosphorylation were selected, which were reached with most of the compounds without an effect of more than 10% on respiration. The concentrations of substances causing such action were then read from the graphs. The actions of these substances on aerobic lactic acid formation have already been compared by a similar method and the values of Table 4 are quoted from the previous study (McIlwain & Grinyer, 1950). The order of potency of the four compounds in bringing about the two changes is seen to be the same. The ratio of active concentrations of any two compounds in depressing phosphorylation is greater than the ratio of concentrations which increase lactic acid formation.

The correlation is thus suggestive only, and not demonstrative of connexion between phosphorylation and glycolysis. The lack of closer correspondence between the two sets of values does not count against the suggestion as there are many difficulties in correlating the actions of substances in slices and homogenates. Some of these have been discussed earlier (McIlwain & Grinyer, 1950). Also, homogenates and slices react differently towards adenosinetriphosphate and cozymase. The degrees of change in aerobic phosphorylation (100 and 200 μg.

Table 4. *Comparison of the concentrations of substances which affect aerobic phosphorylation and glycolysis*

(Derivation of the values is described in the text, this page.)

	Concn. ($\times 10^{-5}$M) decreasing aerobic phosphorylation by		Concn. ($\times 10^{-5}$M) raising rate of aerobic lactic acid formation to	
	100 μg. atom/g./hr.	200 μg. atom/g./hr.	15 μmol./g./hr.	30 μmol./g./hr.
Phenosafranine	0·9	1·6	6	16
Diazine green	1·3	5·6	7	24
Neutral red	130	280	140	550
Nicotine	2200	—	600	1100

atom/g./hr.) and lactic acid formation (15 and 30 μmol./g./hr.) chosen as bases for the comparison were selected for experimental convenience and no opinion is expressed regarding their actual values. Independent techniques are clearly required to show directly any connexion between actions on phosphorylation and glycolysis, and, for this reason, study of the levels of phosphates in tissue slices during their metabolism has been commenced (Buchel & McIlwain, 1950; McIlwain & Cheshire, 1950).

SUMMARY

1. Conditions were studied for obtaining maximum phosphorylation of glucose during respiration of dialysed brain homogenates. Ratios of atoms inorganic phosphorus esterified/atoms oxygen absorbed (P/O ratios) of about 2·5 were obtained.

2. In such homogenates, both phosphorylation and respiration were lowered by chloral, but the P/O ratio changed little, or rose. The P/O ratio was lowered greatly by 2:4-dinitrophenol with little lowering, and sometimes a stimulation, of respiration. Ethyl red had an effect intermediate between those of the above compounds. Phenosafranine also lowered the P/O ratio, with a variable effect on respiration.

3. A system was also devised in which loss of inorganic phosphate and accumulation of labile phosphates catalysed by a brain homogenate could be studied. The homogenate in this case was not dialysed but was more dilute, and adenosine-5-phosphate was added as phosphate acceptor. P/O ratios of 0·6 to 1·8 were observed.

4. 2:4-Dinitrophenol again inhibited phosphorylation with little action on respiration, and azide had a similar effect. The actions of phenosafranine, diazine green, neutral red, and nicotine were also in general similar, though some differences were noted.

5. The findings are discussed in relation to the actions of the different substances on the course of carbohydrate metabolism.

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REFERENCES

Banga, I., Ochoa, S. & Peters, R. A. (1939). *Biochem. J.* **33**, 1980.
Buchel, L. & McIlwain, H. (1950). *Nature, Lond.*, **166**, 269.
Case, E. M. (1932). *Biochem. J.* **26**, 753.
Cross, R. J., Taggart, J. V., Covo, G. A. & Green, D. E. (1949). *J. biol. Chem.* **171**, 655.
Dickens, F. (1936). *Biochem. J.* **30**, 1233.
Dickens, F. (1946). *Biochem. J.* **40**, 145.
Eiler, J. J. & McEwen, W. K. (1949). *Arch. Biochem.* **20**, 163.
Elliott, K. A. C. & Libet, B. (1942). *J. biol. Chem.* **143**, 227.
Elliott, K. A. C., Scott, D. B. McN. & Libet, B. (1942). *J. biol. Chem.* **146**, 251.
Gore, M., Ibbott, F. A. & McIlwain, H. (1950). *Biochem. J.* **47**, 121.
Green, D. E., Loomis, W. F. & Auerback, V. H. (1948). *J. biol. Chem.* **172**, 389.
Johnson, M. J. (1941). *Science*, **94**, 200.
Judah, J. D. & Williams-Ashman, H. G. (1949). *Biochem. J.* **44**, xl.
Keilin, D. & Hartree, E. F. (1937). *Proc. roy. Soc.* B, **122**, 298.
Lardy, H. A. (1945). In Umbreit, W. W., Burris, R. H. & Stauffer, J. F., *Manometric Techniques and Related Methods for the study of Tissue Metabolism*. Minneapolis: Burgess Publishing Co.
Larner, J., Jandorf, B. J. & Summerson, W. H. (1949). *J. biol. Chem.* **178**, 373.
Lipmann, F. (1942). In *Respiratory Enzymes* (Symposium), Wisconsin: University Press.
Lipschitz, M. A., Potter, V. R. & Elvehjem, C. A. (1938). *J. biol. Chem.* **123**, 267.
Loomis, W. F. & Lipmann, F. (1948). *J. biol. Chem.* **173**, 807.
Loomis, W. F. & Lipmann, F. (1949). *J. biol. Chem.* **179**, 503.
Lowry, O. H. & Lopez, J. A. (1946). *J. biol. Chem.* **162**, 421.
McIlwain, H. (1950*a*). *Biochem. J.* **46**, 612.
McIlwain, H. (1950*b*). *Brit. med. Bull.* **6**, 301.
McIlwain, H. & Cheshire, J. (1950). *Biochem. J.* **47**, xviii.
McIlwain, H. & Grinyer, I. (1950). *Biochem. J.* **46**, 620.
Michaelis, M. & Quastel, J. H. (1941). *Biochem. J.* **35**, 518.
Meyerhof, O. (1947). *Arch. Biochem.* **13**, 485.
Meyerhof, O. & Wilson, J. R. (1948). *Arch. Biochem.* **17**, 153.
Ochoa, S. (1941). *J. biol. Chem.* **138**, 751.
Ochoa, S. (1943). *J. biol. Chem.* **151**, 493.
Potter, V. R. (1945*a*). *Arch. Biochem.* **6**, 439.
Potter, V. R. (1945*b*). In Umbreit, W. W., Burris, R. H. & Stauffer, J. F., *Manometric Techniques and Related Methods for the study of Tissue Metabolism*. Minneapolis: Burgess Publishing Co.
Seits, I. F. & Engel'gardt, V. A. (1949). *Dokl. Akad. Nauk S.S.S.R.* **66**, 439. Cited in *Chem. Abstr.* **43**, 7063.
Tepley, L. J. (1949). *Arch. Biochem.* **24**, 383.
Utter, M. F., Reiner, J. M. & Wood, H. G. (1945). *J. exp. Med.* **82**, 217.
Warburg, O. (1926). *Biochem. Z.* **172**, 432.
Warburg, O. & Yabuso, M. (1924). *Biochem. Z.* **146**, 380.

The Inorganic Phosphate and Phosphocreatine of Brain Especially during Metabolism *in vitro*

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As phosphate derivatives form such important intermediates between the functioning of tissues and the metabolism which supports function, we decided to investigate the levels of certain phosphates in brain slices before and during metabolism *in vitro*. Investigation of this type appeared all the more necessary because there is now an extremely interesting group of observations on the levels of phosphorus derivatives in the brains of experimental animals, before and after they have been subjected to various treatments. (For references and an assessment see McIlwain, 1950; some results are quoted in Table 3 of the present paper.) The substances which have been determined following *in vivo* treatment of animals have included inorganic phosphate, creatine phosphate, and adenosine phosphates; and the treatments during which marked changes in these have been observed have included narcosis and induced convulsions. During these processes, the phosphate fractions in which greatest change has been observed have been inorganic and creatine phosphates, and we have accordingly commenced our studies by examining these compounds.

In contrast to the data available from experiments *in vivo*, relatively little information is available of the concentrations of phosphates during *in vitro* metabolism of, for example, brain slices, though Macfarlane & Weil-Malherbe (1941) studied some of these compounds, especially during anaerobic glycolysis, in investigating the course of glycolysis. This deficiency is probably due to the extremely rapid changes which have been reported to take place in these substances, following death or injury to the head. We have fully confirmed the rapidity and magnitude of such changes, but have also found that when brain slices are prepared and maintained under conditions normally regarded as satisfactory for metabolic studies *in vitro*, the changes in phosphates in many cases ceased and could be reversed. Relatively stable levels in inorganic and creatine phosphates were found, which were to some extent characteristic of metabolic conditions, and which could approach those normal to the tissue *in vivo*.

This study includes a subsidiary investigation into procedures for determining inorganic or creatine phosphates in small quantities of tissue. Two methods have been used. The first was an application of the well established calcium-ethanol separations, but determining, as well as phosphates, the creatine of creatine phosphate, precipitated at pH 8·2–8·5 by calcium salts in 80% ethanol. The second depended on the determination of inorganic phosphate under both Fiske & Subbarow's (1925, 1929) conditions and under those of Lowry & Lopez (1946). The difference between the two values gave the phosphates with the lability of phosphocreatine.

Because of its convenience for metabolic studies we have largely employed guinea pig brain cortex during the present work. We have also determined the levels of phosphates *in vivo* in this species by the same methods as employed for the levels *in vitro*.

EXPERIMENTAL

Tissue for phosphates of brain after rapid fixing. (Guinea pigs weighing 250–350 g. were used, fed on a mixed diet.) Trichloroacetic acid was contained in a series of test tube homogenizers, and these were weighed, together with their pestles, and cooled in ice water. To obtain values for normal animals, the whole guinea pig, with minimum preliminary handling, was dropped head first into about 500 ml. liquid O_2 in a 1 quart wide-necked Thermos flask. After about 3 min. it was removed, held with a cloth, hair scraped from the head with a knife, and the skull opened with a $\frac{3}{8}$ in. (10 mm.) firmer chisel already cooled in liquid oxygen. Pieces were chipped from the brain with a cold $\frac{1}{8}$ in. or $\frac{3}{16}$ in. gouge and about 0·2 g. dropped into the trichloroacetic acid of each of the homogenizers. As far as possible, cortex was taken, but dissection was not accurate. Each specimen was homogenized immediately after its addition and the homogenizer returned to ice water. When all specimens had been taken the homogenizers were reweighed, their contents filtered and phosphates determined as described below.

To obtain values after decapitation, animals were guillotined, the head dropped into liquid air after the desired interval, and specimens taken as before.

Tissue for metabolic experiments. An animal was decapitated, the brain removed, and slices of about 0·35 mm. thick cut from the cerebral hemispheres, using a strip of safety-razor blading and a recessed microscope slide as guide. In some cases a Stadie & Riggs (1944) cutter was used. In a few experiments (indicated) the blade was used dry, the slices picked from it with a tared platinum carrier and weighed on a torsion balance before use. Normally, however,

the blade was moistened with the saline used in the later part of the experiment, and immediately after cutting each slice it was floated to a dish of the same saline through which the appropriate gas was passing. They were then picked from the dish with the carrier, adhering saline removed on moist, hardened filter paper or on a porcelain surface, the carrier and slice weighed, and the slice placed in saline in a Warburg vessel or analysed immediately. The times involved in these operations are quoted in the relevant tables. In some cases the slices were weighed at the end of the experiment, after draining on a glass or porcelain surface.

Salines and conditions of metabolism. Glycylglycine-saline contained: NaCl, 0·134 M; KCl, 0·0054 M; KH_2PO_4, $MgSO_4$ and $CaCl_2$, 0·00134 M, and glycylglycine brought to pH 7·4 with NaOH, 0·05 M. Glycine, treated similarly, replaced glycylglycine in a few experiments. Bicarbonate-saline contained: NaCl, 0·124 M; KCl, 0·005 M; KH_2PO_4, $MgSO_4$, and $CaCl_2$, 0·00124 M; $NaHCO_3$, 0·021 M and was gassed with 5% (v/v) CO_2 in O_2. Glucose was used as substrate at 0·012 M and D-glutamic acid, neutralized with NaOH, at 0·02 M.

In most cases the salines (3 ml.) were contained in conical Warburg vessels of approx. 20 ml., the slices added to them, and gas changes followed at 37° by the usual procedures for 15–150 min. The slices were then removed with a mounted, bent platinum wire, drained and sometimes reweighed, and dropped into extracting fluid. Typical experiments involved five or six slices, of which two were controls and others contained the substances under investigation, and in handling such a series each flask was kept in the bath, shaking, until the moment when the slice could be removed and homogenized (see below).

For larger scale experiments in which creatine phosphate was separated, some six or eight slices (total fresh weight about 0·35–0·5 g.) were added to 15 ml. of metabolic medium in a manometric vessel of about 100 ml. At the end of the experiment, any absorbing agent in the centre well was removed, and the slices separated by pouring into a small conical Büchner funnel. They were picked from this with a bent wire, being drained on the porcelain as they were removed. By means of the wire, they were transferred to homogenizer tubes containing deproteinizing agent, already weighed together with their pestles. The tissue was transferred and homogenized as rapidly as possible and after this the tube reweighed to give the weight of tissue.

Determination of inorganic phosphate and phosphocreatine by calcium-ethanol precipitations

These methods have been applied to brain in most detail by Kerr (1935) and Stone (1940, 1943). In the present studies we have adapted such methods to specimens as small as possible, as tissue slices are limited in size. Also, we have determined not only the phosphate distribution on precipitation, but also the creatine content of precipitates obtained under conditions which differentiate creatine phosphate from creatine present as such.

Extraction. Test tube homogenizers of Potter & Elvehjem's (1936) type, made from 150 × 15 mm. tubes, were used. The specimens (0·3–0·5 g.) were rapidly added to cold, 10% (w/v) trichloroacetic acid (1·5 ml.) in a tared homogenizer in ice water, promptly homogenized, and the apparatus reweighed. After leaving for at least 15 min. in ice, the pestles were removed, the tubes centrifuged and the largest convenient quantity (1 ml.) of supernatant removed and measured with a pipette. The tissue weight corresponding to the supernatant taken was calculated from the quantities added to the tube, supposing that the tissue contributed 80% of its weight of water. (The value of 80% was increased when the tissue was known to have absorbed a considerable amount of fluid: see below.) The creatine content of brain obtained by such calculation has been compared with that obtained by successive extractions, and close agreement found.

Inorganic phosphate. The measured portions of supernatants were transferred to further homogenizer tubes calibrated at 3·6 ml. These were already cooled in ice water and 1 drop of 0·1% phenolphthalein was added. With the homogenizer running, solid $Ca(OH)_2$ was added gradually until in slight excess. The pestle was washed with water, making the volume 3·6 ml. Ethanol (0·4 ml.) was then added and the tube left in ice for 15 min. The ethanol had been made just alkaline to phenolphthalein by NaOH. The tube was then centrifuged, the supernatant kept for the determination of creatine phosphate, and the tube drained to leave the precipitate. Inorganic phosphate was determined in the precipitate according to Fiske & Subbarow (see Hawk, Oser & Summerson, 1947).

Creatine of phosphocreatine. This was determined, after conversion to creatinine (see Bonsnes & Taussky, 1945), by the Jaffe reaction. 3·0 ml. of the supernatant after precipitation of inorganic phosphate (and other phosphates) was pipetted to a glass-stoppered tube holding about 20 ml. and calibrated at 10 ml., and 15 ml. of neutralized ethanol added. The supernatants were now adjusted if necessary to the same pH (faintly pink) by NaOH, and left overnight (16–18 hr.) in a refrigerator. In the morning their pH was, if necessary, readjusted with N-NaOH (about 0·01–0·02 ml.) and if adjustment had been made they were left for a further 1 hr. at 0°. The tubes were then centrifuged and the precipitate drained from the supernatant liquid. The precipitate was dissolved in 1·0 ml. 0·2 N-HCl and, if the phosphate of creatine phosphate was to be determined, a sample taken at this point. The portion chosen for creatine determination in the original tube was autoclaved at 15 lb. for 20 min. The tubes were cooled on removal, and 2·0 ml. 1% (w/v) picric acid, followed by 1·0 ml. 5% (w/v) NaOH, was added. The tubes were left 15 min. after mixing, and then made up to the 10 ml. mark with water. They were read against a reagent blank in an absorptiometer using Ilford 604 filters (green, maximum absorption at 520 mμ.).

Phosphate of phosphocreatine was determined in the sample taken, as described above, by Fiske & Subbarow's method.

Determination of inorganic phosphate and phosphates with the lability of creatine phosphate, without chemical separation (method of differential stability)

The method of Fiske & Subbarow (1925, 1929), when applied to biological materials, determines, as well as inorganic phosphate, certain phosphate derivatives which are easily converted to inorganic phosphate by the acid molybdate solution used as reagent. Phosphocreatine is the best known derivative of this type. Kerr (1935) characterized the phosphates of this lability present in trichloroacetic acid filtrates of brain, as creatine phosphate. This was done by the solubility of Ca and Cu salts and their analysis for creatine and P; by the rate of catalysed and non-catalysed hydrolysis; and by isolation of the Ca salt. Application of the method of

Fiske & Subbarow to trichloroacetic acid filtrates of brain preparations will thus determine inorganic plus creatine phosphates, and many of the observations of Tables 2 and 3 have been based on this. Determination of true inorganic phosphate has commonly been made after separation from creatine phosphate by precipitation with Ca or Ba and ethanol similar to that described above.

Lowry & Lopez (1946) have described conditions under which 'true' inorganic phosphate can be determined in the presence of creatine phosphate. They described application of the method to various tissue extracts, including trichloroacetic acid filtrates of brain, and concluded that substances such as acetyl phosphate were absent from the filtrates. Combination of this method with Fiske & Subbarow's appeared to us the simplest way of determining these two substances. We were glad to have also the opinion of Dr M. G. Macfarlane in this connexion. If this combination of methods were applicable to brain slices, it would avoid two Ca-ethanol precipitations and enable smaller quantities of tissue to be handled. We have, in general, found it to be applicable.

In applying Lowry & Lopez's (1946) method to brain extracts, optical density readings with each specimen were taken at 5 and 10 min. as recommended by the authors. The two readings were normally the same. Sometimes a small increase was observed. Usually when this happened, the same increase was found in the readings of the reagent control, so that the corrected readings remained steady; a fresh batch of reagents then commonly gave steady readings with the same brain extracts. In a few cases with extracts of fresh brain a slow increase in values, not paralleled by the control, was found. In these cases the procedure recommended by Lowry & Lopez (1946) was followed: readings were extrapolated to zero time, and the value so obtained regarded as true inorganic phosphate. This correction was necessary in only about 5% of the determinations made.

Application of method to mixture of inorganic and creatine phosphates. In applying their method to trichloroacetic acid filtrates of brain, Lowry & Lopez (1946) recommended dilution of the filtrates to some 150–250 times the volume of the original tissue. We accordingly attempted to apply the combination of methods to concentrations of inorganic and creatine phosphates which might be expected to be present in filtrates diluted to this extent. Creatine phosphate was prepared according to Ziele & Fawaz (1938) and purified by precipitation as Ca salt from ethanol (Fiske & Subbarow, 1929). It still, however, contained a little inorganic phosphate. The solutions indicated in Table 1 were prepared, and their phosphate determined by the two methods. In spite of the small quantities concerned, the recovery of added inorganic phosphate and the determination of creatine phosphate in its presence are seen to be accurate to within 6%.

Phosphates in tissue slices. A series of test tube homogenizers containing 3·3 ml. ice-cold 5% trichloroacetic acid was prepared. As soon as shaking in the manometric experiment stopped, a vessel was taken from the bath, its slice picked on a mounted, bent platinum wire, drained in the flask, dropped into a tube and homogenized. Shaking of the other vessels continued until their slices also were homogenized. Homogenizing occupied only some 10 sec.; the whole operations with six vessels occupied about 7 min. When all slices had been homogenized the mixtures were filtered through fluted papers, in a refrigerator, to test tubes standing in ice.

For inorganic phosphate, 1 ml. filtrate was pipetted into a glass-stoppered test tube in ice, 0·26 ml. N-NaOH (kept in waxed bottle) added to neutralize the trichloroacetic acid, and the volume made to 10 ml. with 0·1 N-sodium acetate of pH 4·0. Of this mixture, 3 ml. was mixed in a cell of a Beckman photoelectric absorptiometer (model DU) with 0·3 ml. of a fresh 1% ascorbic acid solution and 0·3 ml. 1% ammonium molybdate. Determination was completed according to Lowry & Lopez (1946), taking readings at 700 mμ. Corrections were applied for cell characteristics; reagent blanks were normally negligible. A standard was included with each set of determinations, and this was normally very close to a standard curve which had been established at the beginning of the investigation and which was checked at intervals.

For inorganic and creatine phosphate, 1 or 1·5 ml. of the trichloroacetic acid was taken, 1 ml. of the ammonium molybdate and 0·4 ml. of the aminonaphtholsulphonic acid reagents added (Hawk *et al.* 1947), the volume made to 10 ml. with 10% (w/v) trichloroacetic acid, and readings taken after 15 min.

Expression of tissue concentrations

The weights quoted for slices are, unless otherwise described, those after floating the slices in saline and then draining from excess saline. Such slices were known to increase in weight during metabolic experiments (Elliott, 1946; Stern, Eggleston, Hems & Krebs, 1949). The mean increase during typical instances in our experiments were: with glycylglycine buffer, glucose as substrate, aerobically for 75 min., 32%; with bicarbonate buffer and glutamate as substrate, aerobically for 75 min., 36%.

Table 1. *Determination of inorganic and creatine phosphates*

(Solutions of creatine phosphate, without or with the quantities of inorganic phosphate (as KH_2PO_4) indicated, were prepared in 5 ml. portions. 1 ml. was taken for determination according to Fiske & Subarrow and quantities between 0·2 and 2 ml. for determination according to Lowry & Lopez, 1946.)

	P (μg.) determined by		Phosphocreatine-P by difference (μg.)
	Lowry-Lopez	Fiske-Subbarow	
Creatine phosphate *A*	1·65	8·85	7·2
Creatine phosphate + 10 μg. P	12·0	19·5	7·5
Creatine phosphate + 25 μg. P	26·7	33·6	6·9
Creatine phosphate *B*	3·0	20·2	17·2
Creatine phosphate + 10 μg. P	13·9	30·8	16·9
Creatine phosphate + 25 μg. P	28·0	44·2	16·2

These are comparable to those found by Stern *et al.* (1949). We have not determined the weight of the slices at the end of each manometric experiment, because we have found that the slices became more fragile during manometric experiments and to drain them adequately was apt to involve some damage to them, and occupied a time of about 20 sec. during which the slices were not provided with substrate and were at an indefinite temperature. Knowing the lability of phosphates under such conditions, we preferred to drain the slices rapidly (about 2 sec.), but possibly less completely, against the walls of the vessels and not to weigh them.

The expression of concentrations of tissue constituents in terms of the original weight of tissue, which we have adopted, ensures that the quantities of substances are referred to a given weight of metabolizing tissue. We consider that this expression is more significant than one in which they would be referred to a final weight which would include additional material. It is not known how the additional material is associated with the tissue and it is difficult to say whether the swelling should be regarded as intra- or extra-cellular (for some related observations see Dodgson, 1948). If it is extracellular, then the concentrations of phosphate found in the present experiments are directly comparable with those of the fresh tissue *in vivo*; if intracellular, then the concentrations which we quote have been artificially increased by some 33%. But it still remains that they represent exactly the quantity of phosphates which a given original weight of tissue can cause to be kept separate from the salines in which it is metabolizing.

RESULTS

Determination after rapid freezing

Phosphates. Levels of phosphate fractions in brain of several animal species have already been studied. Kerr (1935) first developed adequate methods of fixing the tissue by using liquid air and the value of this method has been confirmed by other workers whose results are quoted in Table 2. Our values for the guinea pigs used in the present studies are seen to be comparable to those found for other small animals. In all determinations, the molar ratio of inorganic phosphate to creatine phosphate is of the order of 1 to 2.

Table 2. *Phosphates of normal brain*

Species (and material)	Reference	Inorganic P (μg./g.)	Phospho-creatine P (μg./g.)	Ratio inorganic P/ phospho-creatine P
Mouse (whole brain)	Stone (1940)	169	101	1·7
Rat (presumably whole brain)	Le Page (1946)	155	70	2·2
	Lindberg & Ernster (1950)	200	101	2·0
Guinea pig (largely cerebral cortex)	Present work: (*a*) eight results, by differential stability	146 (S.D. 26)	83 (S.D. 16)	1·8
Guinea pig (largely cerebral cortex)	(*b*) two results by Ca precipitation	118	102	1·2
Cat (cerebral hemisphere)	Klein & Olsen (1947)	145 (S.D. 54)	74 (S.D. 15)	2·0
	Olsen & Klein (1947*a*, *b*)	140 (S.D. 49)	76 (S.D. 14)	1·8
	Kerr (1935)	125	133	0·9
Dog	Gurdjian, Stone & Webster (1944)	77	97	0·8
Dog	Stone, Webster & Gurdjian (1945)	78	91	0·9
Dog	Kerr (1935)	90	80–120	—

We determined the change in phosphates on decapitation, as the animals from which tissue was taken for metabolic work were commonly killed in this way or by exsanguination. Very rapid changes in the phosphates were found. If the head was put into liquid oxygen about 2 sec. after cutting, creatine phosphate fell to half or a third of its normal value while inorganic phosphate was more than doubled (Table 3). The ratios of inorganic phosphate: creatine phosphate then became greater than 10. Kerr (1935) observed that 90% of the phosphocreatine of rat brain could be lost in 30 sec. and other findings are compared with ours in Table 3.

Creatine of creatine phosphate. Similar changes were found when creatine phosphate was followed by determining the creatine precipitable by calcium salts and ethanol. When intact animals were frozen some 100 μg./g. of phosphocreatine were found (Table 2); after decapitation, about 30 μg./g. The

Table 3. *Changes in the phosphates of brain*

(Percentage changes are calculated on the basis of the average of normal values quoted in Table 2.)

Species and worker		Treatment of animal	Inorganic phosphate		Creatine phosphate		Ratio inorganic P/ creatine P
			μg. P/g.	% change	μg. P/g.	% change	
Mouse	Stone (1940)	Dial	133	−21	136	+35	1·0
Mouse	Stone (1940)	Nembutal	125	−26	138	+37	0·9
Mouse	Stone (1940)	Decapitation	476	+181	27	−74	18
Rat	Le Page (1946)	Nembutal	155	0	93	+33	1·7
Guinea pig	Present work	Decapitation	400	+160	35	−58	11
Dog or cat	Kerr (1935)	Removal of brain in already prepared animal, occupying 3 sec.	—	—	39	−68	—
Dog	Stone *et al.* (1945)	Head injury	200	+160	40	−58	5
		30 min. after death	370	+380	About 4	−96	—

Table 4. *Inorganic and creatine phosphates in freshly cut slices of guinea pig brain cortex*

(Slices were cut from the cortex without additional saline ('dry') or with blade and cutter wet with saline. Tissue, cut or uncut, was left at room temperature until it was precipitated by homogenizing in aqueous trichloroacetic acid. Determinations were made by differential stability.)

Animal	Manner of cutting slice	Time between death and precipitation (min.)	Saline	Time of any sejour in saline (min.)	Inorganic phosphate P (μg./g.)	Creatine phosphate P (μg./g.)	Ratio inorganic P/ creatine P
1	Dry	8	—	—	307	—	—
1	Dry	16	—	—	480	—	—
1	Dry	22	—	—	363	68	5·4
2	Dry	8	—	—	343	—	—
2	Dry	10	—	—	372	60	6·2
2	Wet	17	Bicarbonate-glucose	4	229	37	6·2
2	Wet	22	Bicarbonate-glucose	9	192	10	19·2
3	Wet	21	Glycylglycine-glucose	10	195	40	4·9
3	Wet	21	Glycylglycine-glucose	10	166	21	8·0
4	Wet	23	Glycylglycine-glucose	10	195	24	8·1
4	Wet	23	Glycylglycine-glucose	10	152	33	4·6

values had fallen further by the time that slices had been prepared (Table 5).

Phosphates in freshly cut cortex slices

The inorganic phosphate P of slices cut dry, and fixed in trichloroacetic acid 8–20 min. after death of the guinea pig, was found to be 300–480 μg./g. or some three times that normal to the tissue (Table 4). When the slices were cut moist and left in saline solutions at room temperature for short periods, such as those incidental to metabolic studies, both these values and the values for phosphocreatine were lower. They were also very variable, especially in phosphocreatine (Table 4). Although both values fell, the ratio between inorganic and creatine phosphates remained erratic and several times that observed *in vivo*. During the preparation of slices for metabolic studies, therefore, creatine phosphate has been lost. Inorganic phosphate has been temporarily raised and then lowered to values which, however, remain greater than those normal to the tissue. The level of inorganic phosphate P which was left in the slices (150–230 μg./g.) remained markedly higher than that of the saline in which they had been placed (initially 38·5 or 41·5 μg./ml.; finally about 50 μg./ml.).

Resynthesis of creatine phosphate during metabolism in vitro

This has been demonstrated most clearly by our finding a large increase in creatine precipitable by calcium salts under conditions in which creatine phosphate, but not creatine itself, is precipitated. An equivalent increase in a phosphate with the lability of creatine phosphate was also demonstrated in the same precipitates. Confirmatory evidence was obtained by precipitation with copper salts, and by determination of phosphates by the method of differential stability. Results by the latter method are described in later sections.

Calcium-ethanol precipitation. The specificity of the method, when applied to trichloroacetic acid extracts of brain tissue following *in vitro* metabolism, was first examined. Table 5 shows that when small quantities of creatine, creatinine and creatine phosphate were added to such extracts, only creatine phosphate led to increase in creatine in the 80% ethanol precipitate. Creatine phosphate also led to an equivalent increase in the phosphate determinable by Fiske & Subbarow's method in the precipitates; recovery, judged by either determination, was good. satisfactorily precipitated when added to such extracts. However, we did not find conditions under which a proportion of added creatine was not also precipitated. When the method was applied to simple solutions of comparable quantities of creatine and creatine phosphate, both substances were precipitated. Less creatine was precipitated in the presence of glycylglycine or inorganic phosphate. The application to tissue extracts presumably depends on the presence of associated materials; we have not, therefore, reported results in detail.

Table 5. *Resynthesis of creatine phosphate in brain slices during metabolism* in vitro

(Determinations were by calcium-ethanol separation. The time (quoted) at which the specimen of brain was taken for the initial values, was that at which aeration of the brain slices at 37° was commenced.)

Exp. no.	Treatment	Content (μg. P/g.) of: Inorganic P	Creatine phosphate determined as: P	Creatine phosphate determined as: Creatinine
	Control experiments			
1*a*	Brain, 15 min. post mortem	—	9·9	11·5
b	As *a*, with 1·4 μmol./g. creatine phosphate	—	51·8	53·0
c	As *a*, with 1·6 μmol./g. creatine	—	9·0	12·4
2*a*	Brain, 5 min. post mortem	257	—	15·2
b	As *a*, with 1·6 μmol./g. creatinine	—	—	13·6
	Metabolic experiments			
3*a*	Brain, 15 min. post mortem	192·2	15·5	14·6
b	Slices from *a* after 40 min. respiration in glycylglycine saline	120·9	45·9	42·5
4*a*	Brain, 16 min. post mortem	290·2	—	9·3
b	Slices from *a* after 40 min. respiration in bicarbonate-glucose saline	122	—	41·2
c	As *b*, saline containing $1·5 \times 10^{-4}$ M-creatine	100	—	43·4
d	As *b*, saline containing $1·5 \times 10^{-3}$ M-creatine	102·6	—	45·3
5*a*	Brain, 20 min. post mortem	238·7	—	9·9
b	Slices from *a* after 20 min. respiration in glycylglycine-glucose saline	98·0	—	33·2
c	As *b*, after 70 min.	95·2	—	35·6
6*a*	Brain, 22 min. post mortem	293·6	—	11·8
b	Slices from *a* after 40 min. respiration in bicarbonate-glucose saline (O_2–5% CO_2)	90·8	—	44·9
c	As *b*, anaerobically (N_2–5% CO_2)	108·2	—	9·6

Table 5 also shows that creatine phosphate reappears in slices after *in vitro* respiration under a variety of conditions. The differences under the different conditions are discussed below. Creatine phosphate did not reappear anaerobically or (Buchel & McIlwain, 1950) aerobically in the presence of certain inhibitors.

Precipitation of material in the trichloroacetic acid filtrates as copper salts was also investigated. Fiske & Subbarow (1929) stated this to be especially suitable for differentiating between creatine and creatine phosphate in muscle extracts, and Kerr (1935) applied the findings to brain extracts. In our experiments, copper salts under Fiske & Subbarow's conditions were found to precipitate about the same quantity of creatine from extracts of brain as did calcium-ethanol. Creatine phosphate itself was

Phosphates in slices after metabolism in different solutions

Many determinations by the method of differential stability have shown that the inorganic phosphate and phosphocreatine of slices reach stable levels after *in vitro* metabolism. The data of Table 6 illustrate this. After 75 min. respiration with glucose as substrate in glycylglycine-buffered saline, 15 values are available from slices in independent experiments with different guinea pigs. These gave as mean values for inorganic phosphate P, 95 μg./g. (S.D. 9) and phosphocreatine P, 45·5 μg./g. (S.D. 8). The values at 15, 135 and 195 min. are all close to these averages. The rise in inorganic phosphate and fall in phosphocreatine which occurred shortly after death was thus stopped and even reversed by metabolism *in vitro*.

Further, the ratio of the concentration of inorganic to that of creatine phosphate became about 2·0, close to its value *in vivo*, though the levels of both compounds were lower than those *in vivo*.

The use of bicarbonate as buffer in place of glycylglycine (glucose still as substrate) made only a small change in the levels of phosphocreatine and inorganic phosphate in the slices. The level of inorganic phosphate in all these cases (approx. 100 μg./g.) remained markedly above the level initially present in the saline in which the slices were metabolizing (30–40 μg./ml.).

When glutamate replaced glucose as substrate, with glycylglycine as buffer, characteristic differences were found in phosphate distribution (Table 7). Inorganic phosphate levels became much higher. The values for phosphates with the lability of phosphocreatine became less constant, falling to about 10 μg./g. after 150 min. metabolism. The rate of respiration with glutamate as substrate was some 20–25 % higher than its value with glucose. The effect was also examined of presenting both glucose and glutamate as substrates (glycylglycine as buffer). This gave, at 150 min., levels of both inorganic and phosphocreatine-like phosphates which were characteristic of glucose alone. The rate of respiration was markedly higher than that with either substrate alone (cf. Weil-Malherbe, 1936).

Balance of inorganic phosphate between slices and solution

Throughout the present experiments the level of inorganic phosphate in the metabolic mixture used has been initially at values between 30 and 35 μg. P/ml., or similar to those of blood plasma. The 3 ml. of metabolic mixture of typical experiments thus contained about 100 μg. P, and the slice which was added about 20 μg. P as inorganic phosphate and 1–7 μg. as creatine phosphate. The inorganic phosphate of the medium thus formed a reservoir of phosphate several times greater than that present as inorganic or creatine phosphates in the slice. Nevertheless as the quantity was small in comparison with the quantity of added substrate it was therefore important to follow any changes during the present experiments. We found in all cases an increase in the inorganic phosphate of the medium (Table 8). When expressed in terms of the tissue added this was between 90 and 140 μg. P/g. wet wt. Values for Exp. 3 of Table 5 show that in terms of the observed changes in inorganic and creatine phosphate, slices had lost about 45 μg. P/g. Other experiments showed losses of 60–80 μg./g. The medium thus gained some inorganic phosphate from compounds other than the inorganic and creatine phos-

Table 6. *Phosphates after respiration with glucose in various mixtures*

(All slices cut wet. Time between death and putting slices in appropriate saline, about 10 min.; between death and beginning of manometric experiments, about 35 min. Different values under one set of conditions refer to different experiments on different days. All salines contained glucose at 0·012 M. Determinations: as Table 4.)

Saline	Period of metabolism (min.)	Rate of respiration (μmol./g. wet wt./hr.)	Phosphates (μg. P/g. wet wt.) Inorganic	Creatine phosphate	Ratio inorganic P: creatine P
Glycylglycine	15	68, 69	78, 77	39, 51	1·7
Glycylglycine	75	68·5 (S.D. 4·8)*	95 (S.D. 9·0)*	45·5 (S.D. 8)*	2·1
Glycylglycine	135	65	83	37	2·2
Glycylglycine	195	69	109	50	2·0
Bicarbonate	75	—	115 (S.D. 15)†	44 (S.D. 10)†	2·6
Glycine	75	61, 59	89, 92	29, 30	3·0

* Mean and standard deviation of fifteen experiments. † Of eight experiments.

Table 7. *Phosphates after respiration with glutamate in various mixtures*

(Experimental conditions and recording of results, as in Table 6. D-Glutamate, final concentration 0·02 M.)

Saline	Period of metabolism (min.)	Rate of respiration (μmol./g. wet) wt./hr.)	Phosphates (μg./g. wet wt.) Inorganic	Creatine phosphate
Glycylglycine	75	74, 78, 75	200, 190, 171	30, 34, 39
Glycylglycine	150	73, 80	187, 190	0, 11
Glycylglycine-glucose	150	95	102	54
Bicarbonate	75	—	156	50

Table 8. *Phosphate of medium and slice after metabolism*

(Experimental conditions as in Table 6.)

Medium	Initial moist wt. of slices (mg./3 ml. medium)	Inorganic phosphate P (μg./g. or μg./ml.)		
		Solution, initially	Solution, finally	Slice, finally
Glyclyglycine, glucose	152	33·5	38·6	111
Bicarbonate, glucose	58	31·2	33·0	105
Bicarbonate, glucose	104	30·5	33·4	—
Bicarbonate, glutamate	138	33·5	40·3	156

phates of the slices, but the concentration of inorganic phosphate in the medium was throughout much lower than that of the slice.

DISCUSSION

Resynthesis of creatine phosphate in muscle occurs after its separation from the animal (Meyerhof, 1930). Kerr (1935), however, emphasized that the lability of creatine phosphate in mammalian brain is much greater than in muscle, and the impression gained from the papers quoted in Table 3 is that resynthesis of creatine phosphate in brain *in vitro* would be most unlikely. As various forms of damage and disturbance to the central nervous system lead to lowered creatine phosphate, thin slices of brain might not seem promising material for observation of the resynthesis. Nevertheless, resynthesis has now been demonstrated in respiring slices, and this serves to emphasize the value of tissue-slice techniques and the considerable autonomy of mammalian tissues from a biochemical point of view. Krebs & Eggleston (1949) demonstrated comparable phenomena in brain slices with respect to their maintenance of potassium salts.

It appears likely that the rate of carbohydrate oxidation and glycolysis in tissues and the balance between these two processes are conditioned by the concentrations of labile phosphates and their breakdown products (Belitzer, 1939; Johnson, 1941; for an assessment in relation to brain, McIlwain, 1950). Such rates can be remarkably close in tissue slices to those normal to the tissue *in vitro* (Schmidt, Kety & Pennes, 1945). This is consistent with the restoration which we now observe in the levels of the phosphates investigated. Loss of phosphate from adenosinetriphosphate also occurs in brain after death (Kerr, 1941) and the resynthesis of creatine phosphate *in vitro* is probably preceded by resynthesis of adenosinetriphosphate. The equilibria concerned in brain have been considered by Klein & Olsen (1947) and Meyerhof & Wilson (1947).

The finding of a high rate of glycolysis for a short initial period during aerobic metabolism of brain slices (see, for example, McIlwain & Grinyer, 1950) may be related to their high concentrations of phosphate acceptors and inorganic phosphate at the beginning of such experiments. Because of the possible role of inorganic phosphate in regulating carbohydrate metabolism it is interesting to compare the concentrations which we find in brain slices with those known to affect intermediary metabolism in brain preparations. The concentrations which we find are about 5×10^{-3}M in normal brain, increasing to 12×10^{-3}M with damage. Banga, Ochoa & Peters (1939) found the respiration of brain dispersions to increase with phosphate concentrations especially over the range 1 to 20×10^{-3}M. Long (1945) found a reduction of methylene blue by fumarate to increase with phosphate over the range of about 1 to 5×10^{-3}M, and the formation of pyruvate from fumarate to increase between 1 and 20×10^{-3}M phosphate. It is evident, therefore, that the phosphate concentrations of the tissue are such that increase in them can be expected to bring increased carbohydrate metabolism. This is known also to be sensitive to the breakdown products of the labile phosphates, being increased in brain by creatine (see, for example, Meyerhof & Wilson, 1947) and adenylic acid (see, for example, Case & McIlwain, 1950). Phosphate also activates a glutaminase of brain (Errera & Greenstein, 1949).

The considerable changes in phosphates caused by glutamate appear likely to be related to the effects of this compound on brain respiration (Weil-Malherbe, 1936; Krebs, 1950) and on aerobic glycolysis (Weil-Malherbe, 1938). They are being investigated further. Elliott (1948) has postulated a glutamyl phosphate as intermediate in the synthesis of glutamine in brain, and the evidence of Elliott & Gale (1948) suggests that such a compound may take part in the assimilation of glutamic acid by microbial cells.

The general finding that creatine phosphate is maintained in brain tissue slices has enabled *in vitro* studies to be made of the mode of action of agents which affect the phosphates *in vivo*, as will be reported elsewhere.

SUMMARY

1. The inorganic phosphate and phosphocreatine in samples of about 0·5 g. of guinea pig brain have been determined after calcium-ethanol separation. Both phosphates and creatine have been estimated. Inorganic phosphate and phosphates with the lability

of phosphocreatine have been determined in specimens of about 0·1 g. brain without chemical separation.

2. After death, a very rapid fall in phosphocreatine and rise in inorganic phosphate takes place in the brain of guinea pig as of other species.

3. On slicing brain cortex and allowing the slices to respire with glucose under normal metabolic conditions, the inorganic phosphate falls and the phosphocreatine rises until the concentrations approach those normal to the tissue *in vivo*.

4. The concentrations reached vary with the metabolic mixture.

REFERENCES

Banga, I., Ochoa, S. & Peters, R. A. (1939). *Biochem. J.* **33**, 1109.
Belitzer, V. A. (1939). *Enzymologia*, **6**, 1.
Bonsnes, R. W. & Taussky, H. H. (1945). *J. biol. Chem.* **158**, 581.
Buchel, L. & McIlwain, H. (1950). *Nature, Lond.*, **166**, 269.
Case, E. M. & McIlwain, H. (1950). *Biochem. J.* **48**, 1.
Dodgson, M. C. H. (1948). *Nature, Lond.*, **162**, 253.
Elliott, K. A. C. (1946). *Proc. Soc. exp. Biol., N.Y.*, **63**, 234.
Elliott, W. H. (1948). *Nature, Lond.*, **161**, 128.
Elliott, W. H. & Gale, E. F. (1948). *Nature, Lond.*, **161**, 129.
Errera, M. & Greenstein, J. (1949). *J. biol. Chem.* **178**, 495.
Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
Fiske, C. H. & Subbarow, Y. (1929). *J. biol. Chem.* **81**, 629.
Gurdjian, E. S., Stone, W. E. & Webster, J. E. (1944). *Arch. Neurol. Psychiat., Chicago*, **51**, 472.
Hawk, P. B., Oser, B. L. & Summerson, W. H. (1947). *Practical Physiological Chemistry*, 12th ed. London: Churchill.
Johnson, M. J. (1941). *Science*, **94**, 200.
Kerr, S. E. (1935). *J. biol. Chem.* **110**, 625.
Kerr, S. E. (1941). *J. biol. Chem.* **140**, 77.
Klein, J. R. & Olsen, N. S. (1947). *J. biol. Chem.* **167**, 747.
Krebs, H. A. (1950). *Biochim. Biophys. Acta*, **4**, 249.
Krebs, H. A. & Eggleston, L. V. (1949). *Biochem. J.* **44**, vii.
Le Page, G. A. (1946). *Amer. J. Physiol.* **146**, 267.
Lindberg, O. & Ernster, L. (1950). *Biochem. J.* **46**, 43.
Long, C. (1945). *Biochem. J.* **39**, 143.
Lowry, O. H. & Lopez, J. (1946). *J. A. biol. Chem.* **162**, 421.
Macfarlane, M. G. & Weil-Malherbe, H. (1941). *Biochem. J.* **35**, 1.
McIlwain, H. (1950). *Brit. med. Bull.* **6**, 301.
McIlwain, H. & Grinyer, I. (1950). *Biochem. J.* **46**, 620.
Meyerhof, O. (1930). *Die Chemischen Vorgänge im Muskel.* Berlin: Springer.
Meyerhof, O. & Wilson, J. R. (1947). *Arch. Biochem.* **14**, 71.
Olsen, N. S. & Klein, J. R. (1947*a*). *J. biol. Chem.* **167**, 739.
Olsen, N. S. & Klein, J. R. (1947*b*). *Arch. Biochem.* **13**, 343.
Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.
Schmidt, C. F., Kety, S. S. & Pennes, H. H. (1945). *Amer. J. Physiol.* **143**, 33.
Stadie, W. C. & Riggs, B. C. (1944). *J. biol. Chem.* **154**, 687.
Stern, J. R., Eggleston, L. V., Hems, R. & Krebs, H. A. (1949). *Biochem. J.* **44**, 410.
Stone, W. E. (1940). *J. biol. Chem.* **135**, 43.
Stone, W. E. (1943). *J. biol. Chem.* **149**, 29.
Stone, W. E., Webster, J. E. & Gurdjian, E. S. (1945). *J. Neurophysiol.* **9**, 233.
Weil-Malherbe, H. (1936). *Biochem. J.* **30**, 665.
Weil-Malherbe, H. (1938). *Biochem. J.* **32**, 2257.
Ziele, K. & Fawaz, G. (1938). *Hoppe-Seyl. Z.* **256**, 193.

The Reaction of Oxidizing Agents with Wool

3. THE INFLUENCE OF THE MORPHOLOGY ON THE RATE OF REACTION

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A detailed study of the kinetics of the reaction between wool and aqueous chlorine solutions at pH 2 by Alexander, Gough & Hudson (1949) has shown that the rate is controlled either by diffusion through a film of liquid surrounding the fibre or by diffusion within the fibre, depending on the conditions of treatment. The rate of the reaction increases with increasing agitation up to a limiting stirring rate, after which it becomes constant. At this point the temperature coefficient changes sharply, showing that a change in mechanism has occurred. The following observations show that the rapid rate at high stirring speeds with chlorine at pH 2 is not governed by the actual chemical reaction but by the diffusion of the chlorine to the site of reaction where it reacts instantaneously:

(1) The quantity of chlorine which has reacted increases with time according to a parabolic law which is to be expected for diffusion into the fibres under the experimental conditions employed.

(2) The observed activation energy (12·5 kg. cal./mol.) is the same as that found for a wide variety of

reactions, e.g. dyeing, ion exchange (Alexander & Hudson, 1950) and oxidation with acid potassium permanganate (Alexander & Hudson, 1949). The one feature common to all these processes is diffusion within the fibre.

(3) The reaction between chlorine in acid solution and the amino-acids cystine and tyrosine, which are preferentially oxidized in wool, is extremely rapid compared with the rate of reaction with wool.

(4) The rate of reaction with wool in which all the disulphide bonds have been changed to —C—S—C— linkages is the same as that for untreated wool in which the disulphide bond is oxidized preferentially (see p. 24).

(5) On interrupting a kinetic run, a break in the curve is observed which is typical for diffusion-controlled processes (see p. 23).

The reaction of chlorine in acid solution is very similar to the oxidation by potassium permanganate at pH 2 (Alexander & Hudson, 1949), and in both cases diffusion appears to proceed through a homogeneous medium. Complete penetration of chlorine occurs, as is shown by the removal of all the cystine in acid solutions. The fibre behaves as a uniform gel of relatively low water content, although the actual fibre is thought to consist of a complex network of small micropores filled with solvent through which diffusion occurs. This behaviour of the fibre towards the diffusing molecules is interesting as the morphological structure of wool is known to be extremely complex, consisting of various layers differing in accessibility (see below). It will be shown that the diffusion of chlorine and permanganate in alkaline solution is affected by the variation of structure within the fibre, which, however, does not affect diffusion from acid solution.

EXPERIMENTAL METHODS

The measurement of the rate of reaction has been described in detail elsewhere (Alexander, Gough & Hudson, 1949) and will only be summarized here. Purified wool samples, after immersion in the appropriate buffer solution for 24 hr., were fitted uniformly round a frame stirrer and rotated at known speeds in a known volume of Cl_2 solution at the required pH. Samples of solution were withdrawn periodically and analysed iodometrically. Reactions at pH <8 were conducted in sealed flasks, but in alkaline solution open beakers were employed as no loss of Cl_2 occurs. The following buffer solutions of ionic strength, approx. 0·1, maintained the pH of solution constant during the reaction:

pH 3 and 4. Potassium hydrogen phthalate and H_2SO_4.
pH 5 and 6. Sodium acetate and acetic acid.
pH 7–10. KH_2PO_4 and NaOH.
pH 11. 0·1 M-Na_2CO_3.

The volumes of the buffer components used depended on the alkalinity of the standard NaClO stock solution.

In stating the concentrations of the reactants, three variables must be specified, namely, the quantity of Cl_2 in solution, the volume of solution and the weight of fabric used. In the work described here, the ratio of weight of fibres to volume of solution was kept constant at 1 : 150 and the concentration of Cl_2 is expressed as percentage by weight of the weight of wool.

The wool was pretreated in various ways in several of the experiments as follows:

Potassium cyanide. The wool fabric was first wetted out in water overnight and then immersed in a 0·7% solution of KCN and boiled for 30 min. The fabric was next washed until no further reaction for CN^- ions was observed in the washings, and finally placed in the appropriate buffer for 24 hr. According to Cuthbertson & Phillips (1945) the cystine is converted almost quantitatively to lanthionine by this treatment. It was found that in the present case the cystine content of wool decreased from 10·08 to 0·72%, corresponding to a 93% loss of cystine.

Chlorine at pH 2. The wool, previously wetted out in water, was allowed to react with a solution of Cl_2 (5 and 10% of the weight of wool) in dilute H_2SO_4 at pH 2, until no Cl_2 remained in solution. After washing well in running water the fabric was left overnight in the pH 10 buffer.

Partial acid hydrolysis. The samples of wool were sealed in tubes containing N-HCl and placed in an electric oven at 80°. After 8 hr. the samples were withdrawn and wetted out in the buffer at pH 10.

Potassium hydroxide. Samples of fabric (2 g.) were dipped for 30 sec. into a solution of 2·5 g. KOH in 1000 ml. of white spirit and 100 ml. of *n*-butanol at room temperature. Under these conditions reaction is confined to the extreme outside of the fibres. The samples were then washed in running water and immersed overnight in the buffer at pH 10.

RESULTS

Effect of pH *on rate*

It is observed from Fig. 1 that the rate of reaction remains almost constant from pH 2 to 7, but rapidly decreases from pH 8 to 11. In the absence of a more

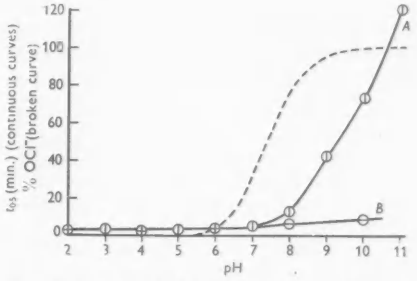

Fig. 1. Effect of the pH of the Cl_2 solution on the rate, given as half-life value ($t_{0.5}$), for untreated (*A*) and KCN-treated wool fibres (*B*). Broken curve: effect of pH on the concentration of OCl^- ions, given as percentage of oxidizing agent present. Stirring rate 600 rev./min.; temp. 0°; 10 g. Cl_2/100 g. wool.

satisfactory method of comparing rates of reactions proceeding by different mechanisms the half-life values are used. The change in composition of the

chlorine solution with pH is also included in the graph and is seen to run parallel with the decrease in rate. The constant rate of reaction in the pH range 2–7, where the ratio of chlorine to hypochlorous acid changes considerably, is explained by the similar diffusion coefficients of the two species, as the reaction is controlled by diffusion within the fibre. In addition, the penetration of neutral molecules would be unaffected by electrical changes on the surface and within the fibre, except indirectly by the change in swelling with pH of solution. That the half-life values remain almost constant shows that swelling has a negligible effect. Above pH 6, however, the solution contains increasing proportions of hypochlorite ions, so that over the pH range 7–9 the reaction is probably governed by the concentration of hypochlorite ions and hypochlorous acid. It appears from Fig. 1 that the rate of reaction with the hypochlorite ions is slow compared with the hypochlorous acid reaction. It has been shown that the rate is first order with respect to the initial chlorine concentration in acid solution (Alexander, Gough & Hudson, 1949). Thus, if it is assumed that the rate of the reaction with hypochlorous acid is greater than that of the hypochlorite ion reaction, approximate rate constants can be calculated neglecting the latter reaction as follows:

$$k = k_0\,[\mathrm{HOCl}],$$

where k = observed velocity constant and k_0 = velocity constant with 100 % HOCl in solution. Table 1 includes such values for pH 6–9. Thus, at pH 7 and 8 reaction proceeds mainly by diffusion of hypochlorous acid through the fibre, but for pH values greater than 8 the rate of reaction is greater than it would be if hypochlorous acid alone reacts.

Table 1. *Comparison of observed half-life values with those calculated on the assumption that the reaction is due to hypochlorous acid alone*

(Temp. 0°.)

pH	HOCl (%)	$t_{0.5}$ (obs.) (min.)	$t_{0.5}$ (calc.) (min.)
6	97	3·1	3·1
7	67	4·5	4·5
8	23	12·0	13·0
9	3	43·0	100·0

The properties of the treated wool and the nature of the reaction products support the conclusion from this kinetic evidence that the reaction at pH 8 is controlled mainly by reaction with hypochlorous acid. Thus, reaction at pH 8, but not at pH 10, leads to the complete conversion of cystine to cysteic acid at pH 2 (Alexander, Hudson & Fox, 1950). The extent of damage to the fibre, measured by decrease in strength, solubility in alkali and removal of scale structure, is of the same order for samples treated at pH 2 as at pH 8, whereas it is much less if the wool sample is chlorinated at pH 10. In addition, wool treated at pH 2 and 8 is resistant to shrinkage, but fabric treated at pH 10 felts almost to the same extent as an untreated sample (Alexander, 1950).

At pH 9–11 the solution is composed almost entirely of hypochlorite ions, but the rate of reaction decreases continually. In this pH range, therefore, reaction is due entirely to the hypochlorite ion and the rate is highly pH-dependent. This process is discussed further in the final section.

Effect of stirring

The change in rate with pH is reflected in the effect of stirring on reaction rate at various acidities. Thus above pH 8 the reaction is found to be almost independent of stirring, although below pH 8 the

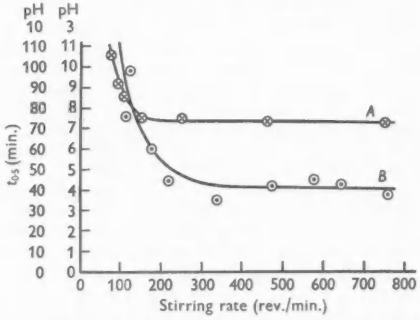

Fig. 2. Effect of stirring on the rate of reaction ($t_{0.5}$) of Cl_2 solutions at pH 3 (*A*) and pH 10 (*B*); 10 g. Cl_2/100 g. wool; temp. 0°.

rate is highly dependent on stirring (Fig. 2). Thus, if diffusion is the rate-determining step in alkaline solution, the diffusion of hypochlorite ions through the fibre must be slower than the diffusion in solution to the surface and also much slower than the diffusion of chlorine and hypochlorous acid through the fibre. It is observed that at pH 7 and 8, when reaction may be controlled by hypochlorous acid and hypochlorite ions, the rate is more dependent on stirring than at pH 9 but less so than at pH 2–6. In addition, with low concentrations (0·5 % on weight of wool or 0·001 N) at pH 10 the reaction becomes more dependent on stirring, showing that here the rate may be partly dependent on the rate of diffusion of ions to the surface.

Effect of concentration

Change in concentration affects the rate of reaction in different ways at pH 2 and 10 (Table 2). At pH 2 the rate suddenly decreases for concentrations less than 10 % chlorine and remains con-

stant, which shows the change in mechanism from diffusion through the fibre to diffusion in solution.

Table 2. *Effect of concentration of chlorine on rate of reaction at various* pH *values at* 0° *and* 600 *rev./min. stirring*

(Fibres, 1 g. in 150 ml. of solution.)

pH	Cl_2 concn. (g./100 g. wool)						
	25	15	10	7	3	1	0·5
	$t_{0·5}$ (min.)						
2	3·2	2·5	2·7	—	1·6	1·7	1·5
4	—	—	3·4	—	2·0	2·0	2·1
8	—	—	11·5	—	5·2	4·6	4·9
10	60·0	—	71·0	65	57·0	44·0	30·0

At pH 10, however, the half-life values ($t_{0·5}$) are approximately constant for concentrations greater

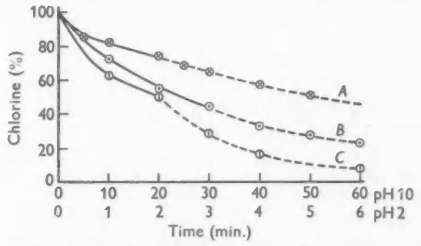

Fig. 3. Rate curves for experiments at 600 rev./min. and 0° for the following treatments: *A*, 4 g. Cl_2/100 g. wool at pH 10 interrupted after 20 min.; *B*, 0·5 g. Cl_2/100 g. wool at pH 10 interrupted after 30 min.; *C*, 10 g. Cl_2/100 g. wool at pH 2 interrupted after 2 min.

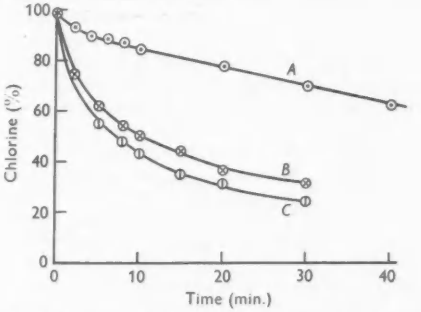

Fig. 4. Rate curves at pH 10 with 10 g. Cl_2/100 g. wool at 600 rev./min. and 0°. *A*, untreated wool; *B*, pretreated with 10 g. Cl_2/100 g. wool at pH 2 (curve for pretreatment with 5 g. Cl_2/100 g. wool is identical); *C*, pretreated with KCN.

than 5 % chlorine, showing that the rate is proportional to the concentration. For lower concentrations the value of $t_{0·5}$ decreases continuously showing that the rate of reaction becomes almost independent of the initial chlorine concentration. An examination of the form of the rate curves for various concentrations leads to an explanation of this change in $t_{0·5}$. The rate curve at pH 10 (see Fig. 3*B* and 4*A*) consists of two distinct portions corresponding to an initial rapid reaction followed by a slower process which forms the major portion of the reaction. The range of the initial rapid reaction is seen to increase with decreasing concentration, and can be detected with difficulty with 10 % chlorine (Fig. 4*A*). This corresponds to an approximately constant quantity of chlorine, independent of initial concentration, and is most likely due to adsorption on and reaction with the surface and near surface of the fibres. From the curve for 0·5 % chlorine, assuming the value of 10^4 sq.cm./g. for the surface area obtained from adsorption data (Rowen & Blaine, 1947) it is found that if the initial reaction is confined to the surface, each hypochlorite ion is accommodated in approx. 10 A.^{2}. This reasonable figure shows that the initial limited portion of the rate curves can be accounted for by reaction confined to the surface.

The major portion of the rate curve for concentrations greater than 1 % chlorine is found to follow a logarithmic law (Fig. 5; see also Discussion, p. 25),

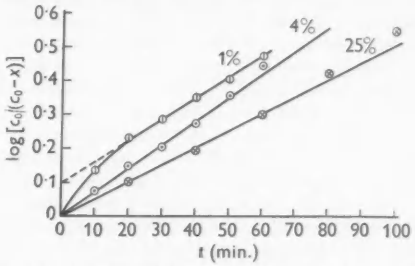

Fig. 5. Graphs of $\log [C_0/(C_0 - x)]$ against t at 0° for 1, 4 and 25 g. Cl_2/100 g. wool at pH 10; stirring at 600 rev./min. C_0 is the initial concentration of OCl^- ion and $(C_0 - x)$ the concentration at time t.

which shows that the rate of reaction is proportional to the hypochlorite concentration. In support of this it is found that the curves plotted in the form of percentage decomposition against time can almost be superimposed if allowance is made for the initial rapid reaction. It is, therefore, the influence of the initial surface reaction which causes the continual increase in $t_{0·5}$ values with increasing concentration, reaching a constant value when the initial reaction is negligible.

Activation energies

Table 3 includes values of the apparent activation energies (E) for pH 9 and 10 calculated from the simple Arrhenius relation. For the more concentrated solutions, these values are calculated from $t_{0·5}$ values, whereas in the case of the very dilute

solutions E was calculated from $t_{0.5}$ and from initial rates, identical values being obtained. This indicates that the initial reaction and the major reaction are subject to the same restrictive influences. The average value of 10·4 kg. cal./mol. is somewhat less than the corresponding value in acid solution (12·5), which is additional proof that different mechanisms operate in acid and in alkaline solution.

Table 3. *Experimental activation energies for reaction at* pH 9 *and* 10

pH	Cl (%)	Temp. (°)	$t_{0.5}$ (min.)	E (kg. cal./mol.)
9	0·5	0	11·6	10·9
		17	3·6	
	4·0	1·5	33·0	10·5
		16·9	12·0	
10	0·5	0	31·0	10·0
		19·5	8·9	
		25·9	6·1	
	4·0	0	60·0	10·4
		19·5	19·7	
		25·9	11·2	

Interrupted runs

In some experiments at pH 2 and 10, the piece of fabric was withdrawn from the solution after a given time and kept out of contact with the solution for sufficient time to allow any diffusion gradient to decrease considerably. The fabric was then rapidly immersed in the solution and the rate of reaction determined as before. If the reaction is controlled by diffusion in the fibre, the rate on continuation will be greater than the rate immediately prior to the interruption, due to the establishment of a large diffusion gradient. Such behaviour has been observed in several cases, e.g. the reaction between potassium permanaganate with wool at pH 2 (Alexander & Hudson, 1949), anion exchange (Kunin & Myers, 1947) and cation exchange (Kressman & Kitchener, 1949). The results of similar experiments are shown in Fig. 3, in which typical examples at pH 2 and 10 are compared. It is noted that with 10 % chlorine at pH 2 a definite break in the rate curve is obtained, thus providing further evidence that this reaction is controlled by diffusion through the fibre. At pH 10, however, no discontinuity is observed, even for an initial concentration of 0·5 % chlorine, which is also the case in the oxidation of wool by potassium permanganate at pH 9·2. These observations show that in alkaline solution the rate of oxidation is not controlled by diffusion through the fibre treated as a homogeneous medium.

Preliminary treatments

Significant results were obtained by pretreating the fibres with a variety of reagents known to modify various groups in the wool. Thus, treatment with boiling potassium cyanide solution increases the rate of reaction with hypochlorite solution at pH 10 considerably (Fig. 1). Although the rate is greatly increased at pH 9 and 10, it is unaffected by pretreatments at pH 2, 4 and 6, which also supports the conclusion that the rate in acid solution is controlled by diffusion through the fibres. The increase in rate at pH 9 and 10 is not due specifically to the removal of cystine, as it is observed that acid hydrolysis without attack on disulphide bond increases the rate (see Table 4).

Table 4. *Effect of various pretreatments on the subsequent rate of chlorination with* 10 % *chlorine at* 0° *and* pH 10

(Pretreatment at room temperature unless otherwise stated.)

Treatment	$t_{0.5}$ (min.)
Untreated	71
N-HCl, 80° for 8 hr.	5·2
5 % Cl_2, pH 2	10·4
10 % Cl_2, pH 2	10·0
KOH in butanol for 30 sec.	64·0

In addition, it is known that chlorine at pH 2 also reacts with the amino groups of the fibre, and eventually with the polypeptide chain. Treatment with this reagent increases the rate of subsequent chlorination at pH 10 considerably. Finally, experiments with potassium permanganate at pH 1 and 9·2 show that even mild treatments in acid solution increase the rate of reaction with sodium hypochlorite solution at pH 10. Pretreatment with alkaline permanganate solution, however, has no effect on the rate (see Table 5). Although reaction with permanaganate at pH 1 is extensive, only 25–30 % of the total cystine can be oxidized (Alexander, Hudson & Fox, 1950) so that modifi-

Table 5. *Effect of pretreatment with potassium permanganate solutions at* 25° *on the subsequent rate of chlorination with* 10 % *chlorine at* pH 10 *and* 25°

$KMnO_4$ concentration (g./100 ml.)	Time of pretreatment	$t_{0.5}$ (min.)
	Solution containing N-H_2SO_4	
0	0	14
0·1	2 min.	10
0·1	10 min.	4
0·5	1 min.	4
0·5	5 min.	4
1·0	10 sec.	6
1·0	1 min.	4·5
	Solution at pH 9·2 (borax buffer)	
0·1	1 hr.	15
0·1	18 hr.	13
0·5	5 min.	15
0·5	30 min.	11

cation of the disulphide bond alone is not responsible for these greatly increased rates. Pretreatment with ethanolic potassium hydroxide, which was shown by Lindberg, Philip & Gralén (1948) to remove the very thin outermost layer of the wool (the epicuticle), does not influence the rate of alkaline chlorination (Table 4).

DISCUSSION

The experimental data presented here and in previous papers shows that the rate-determining process of the reaction of wool with oxidizing agents in acid solution is the diffusion of molecules through the solid to reaction sites, accompanied by rapid reaction. The penetration of diffusing molecules is thought to proceed through channels of molecular dimensions containing solvent, and to be retarded by the molecular chains of the fibre leading to a high activation energy of the process. Although the amphoteric nature of protein fibres results in changes in the molecular structure and distribution of electrical charges within the fibre with change in pH of the solution, the kinetics of reaction at pH 2–7 can be interpreted as isotropic diffusion through a uniform gel.

The rate of reaction decreases continually from pH 8 to 11, although the available chlorine exists almost totally as hypochlorite ions. This decrease in rate is accompanied by an increase in neutralization of amino groups (approx. 25% neutralized in the presence of 0·1M-electrolyte, the buffer concentration used at pH 11; see Steinhardt & Harris, 1940), which suggests that the reduced rate is due to electrical repulsion between negative carboxyl ions and hypochlorite ions diffusing within the fibre. The experimental results, discussed in the previous section, show that the rate is not controlled by diffusion through a uniform medium. Most of the kinetic data would be explained if the rate in alkaline solution were governed by the actual chemical reaction. In this case the rate would probably be of the first order so long as the wool was in considerable excess; the rate would be independent of stirring; no breaks in the interrupted curves would be expected; and the energy of activation could well be less than that of the fibre diffusion-controlled process at pH 2. This possibility is not substantiated by the following considerations. The kinetics of oxidation with alkaline permanganate solution are very similar to the kinetics of alkaline chlorination. Thus, on changing the pH from 2 to 9·2, the rate of reaction is greatly reduced, and becomes almost independent of stirring. The kinetic curve changes from parabolic to logarithmic, and in interrupted treatments there is no discontinuity at pH 9·2, although this is very marked at pH 2. This close similarity between the effects of the two dissimilar oxidizing agents suggests that the rate is not controlled by chemical decomposition. Moreover, the two reagents react quite differently with wool both in acid and in alkaline solution (Alexander, Hudson & Fox, 1950), e.g. alkaline permanganate does not react with the disulphide bond, unlike alkaline hypochlorite.

The data on the rate of alkaline chlorination after the various pretreatments are difficult to interpret if the rate is governed by chemical reaction. Thus, if the increased rate is due to general degradation with an increased number of available sites for reaction, the effect of a pretreatment at pH 2 with 10% chlorine (when 43% cystine has reacted) would be expected to be greater than that with 5% chlorine (when 22% cystine has reacted). The results in Table 4 show that this is not the case. Similarly, both a mild and a severe acid permanganate pretreatment accelerate a subsequent alkaline chlorination to the same extent. This indicates that modification of the outermost portion of the fibres is a major factor influencing the rate, and moreover the observed kinetics could be explained if the rate-controlling step were diffusion through a surface obstruction. Recent researches on the morphology of the wool fibre have established the presence of a membrane near the surface which indicates the validity of such a treatment.

Various workers (cf. Zahn, 1950) have produced evidence for the existence of a continuous tube-like membrane ('subcuticle') extending the whole length of the fibre, and variously estimated as representing 2–5% of the weight of the wool fibre. As a result of microscopic examination, the material was thought to be located in the fibre between the outer scales and the cortical cells. However, the methods of isolation involved severe degradation and its identification as a specific unit within the fibre was doubtful. Lindberg *et al.* (1948) have detected, with the aid of the electron microscope, a thin layer about 100 A. thick (i.e. approximately 0·1% of the wool as a whole) on the surface of the fibre and called this the 'epicuticle'. The epicuticle, which is probably fatty material, can be largely removed by prolonged ether extraction, or more satisfactorily by rapid immersion in potassium hydroxide in white spirit. Removal of the epicuticle increases the rate of adsorption of acids, although it is shown in the previous section that the rate of the alkaline hypochlorite reaction is not affected by the potassium hydroxide treatment. The epicuticle does not, therefore, affect the rate of penetration of hypochlorite ions and removal of the epicuticle is not responsible for the increased rate after various pretreatments.

Mercer, Lindberg & Philip (1949) questioned the existence of the subcuticle as isolated by Zahn and others, and believe that the tubes were a mixture of epicuticle and underlying scales in varying degrees of degradation. The existence of a protein component of increased chemical resistance, representing 7–10%

of the wool in the form of a tube similar to the subcuticle of Zahn (1950), was established by Alexander & Earland (1950) as follows. The fibres were treated with peracetic acid, which reacts in wool almost exclusively with cystine until all the cystine had been oxidized. On subsequent treatment with ammonia the major part of the fibres dissolved, leaving a 7–10 % residue in the form of cylindrical tubes identical in external form with the original fibres. An X-ray photograph of the soluble protein in powder form, obtained by precipitation from the ammoniacal solution with dilute acid, shows this to be in the α-configuration, whereas the insoluble tubes give a β-picture (Alexander, Earland & Happey, 1950). The β-fold provided a much stronger structure than the α-material because of the closer packing and better inter-chain hydrogen bonding, and probably protects the elastic interior of the fibre.

The position of this membrane with respect to the scales cannot be determined from visual and electron microscopic observation and the term 'subcuticle' may well be misleading. It is certain, however, that it represents the major part of the highly complex cuticle and it seems possible that it is a skin below the scales which surrounds the cortex. For the purpose of interpreting the kinetics of oxidation with alkaline chlorine and alkaline permanganate solutions, the fibres will be treated as a homogeneous gel surrounded by a resistant membrane. That the resistant membrane lies close to the fibre surface is shown by the observation (see p. 23) that the preliminary rapid surface reaction constitutes only about 1 % of the total possible reaction.

If the hypochlorite ions do not modify the membrane during the course of the reaction, the rate of transport P across the membrane of thickness l and area of membrane permeable to hypochlorite ions A_m is given by

$$P = A_m D/l\,(C_l - C_m),$$

where C_l and C_m are the concentrations of free hypochlorite ions on either side of the membrane and D the diffusion coefficient. If it is assumed that diffusion and reaction within the fibre are rapid processes (i.e. $C_m \sim 0$) and C_l is equal to the hypochlorite ion concentration in solution, then

$$D = \frac{lV}{A_m t} \ln \frac{C_0}{C_0 - x},$$

where V is the volume of solution, C_0 the initial concentration and $(C_0 - x)$ the concentration at time t. This logarithmic relation between time and concentration is obeyed approximately in alkaline solutions (Fig. 3). In a process of this type, no discontinuity in the rate curve would occur on interrupting a reaction.

Damaging or opening the structure of the membrane must lead to an increase in the rate of reaction with alkaline hypochlorite as the available area permeable to the ions A_m is increased, which explains the effect of the different pretreatments. The prevention of felting shrinkage in wool by oxidizing agents is caused by disruption of the cuticle which need not necessarily be removed (Speakman & Goodings, 1926; Alexander, 1950). It would appear more than a coincidence that acid chlorine and acid permanganate solutions, which both effectively reduce the tendency of wool to felt, are effective in increasing the rate of reaction of alkaline hypochlorite, unlike alkaline permanganate and, of course, alkaline hypochlorite itself, which do not prevent felting (Alexander, Carter & Hudson, 1949).

Pretreatment with potassium cyanide, leading to the rupture of all the disulphide bonds, and partial hydrolysis both open up the membrane, thus rendering it more permeable, and hence increase the rate of reaction with alkaline hypochlorite. In acid solution chlorine initially attacks the membrane which then no longer restrains the diffusion, so that movement within the bulk of the fibre becomes the rate-controlling step. As the retardation of the membrane is due to its electrical charge which repels like charged ions, the preliminary diffusion of uncharged molecules such as chlorine and hypochlorous acid is not reduced by the presence of the membrane, so that diffusion appears to proceed through a homogeneous medium.

The driving force of the diffusion process is the concentration gradient of the hypochlorite ions, which is maintained at a high value by the complete removal on one side of the membrane due to chemical reaction. The negative membrane potential (Teorell, 1949), which cannot be measured directly, opposes the diffusion, but remains approximately constant as the solution in contact is maintained at constant pH during the reaction. In addition, chloride ions resulting from the reduction of the hypochlorite ions diffuse out at the same rate as the latter diffuse in and therefore no accumulation of ions can modify the charge on the membrane.

The continual decrease in the rate of chlorination with increasing pH after the ionization of hypochlorite is complete shows that the retarding influence of the membrane is due to electrical interaction between diffusing anions and the charged carboxyl groups in the membrane. As the pH is increased the amino groups become unionized (i.e. OH^- ions are combined) and the fibre becomes negatively charged by the carboxyl groups which had previously formed part of a salt link. There is some evidence (Alexander & Kitchener, 1950) that the outside groups of the fibre titrate preferentially, so that the charge on the membrane at a given pH may be greater than is indicated from the overall titration curve.

The individual negative charges within the fibre do not extend over great distances as they are neutralized by an electrical double layer containing an excess of cations (e.g. Na^+). The fibre is therefore freely permeable to anions so long as the number of free carboxyl groups is small, as is shown by the fact that with cyanide-treated wool, in which the membrane has been opened up, the rate of chlorination is the same at pH 8 as at pH 2. At pH 10 electrical interaction between the anions and the wool begins to be detectable and the rate of chlorination of cyanide-treated wool decreases.

SUMMARY

1. The kinetics of the reaction of wool with aqueous chlorine solution of pH less than 8 are explained by the uniform diffusion of chlorine or hypochlorous acid through the swollen fibres. It has been shown that this process is not rate-determining in solutions of pH greater than 8.

2. The form of the kinetic curve, the effect of various pretreatments on the rate, and the temperature coefficient of the process, all show that the alkaline reaction is not controlled by the actual oxidation process, but can be interpreted in terms of diffusion across a surface barrier.

3. Recent morphological researches on wool fibres have established the existence of two membranes, one of which, the epicuticle, is situated on the extreme outer surface, whilst the other forms part of the cuticle and is referred to as the subcuticle. It is shown that the presence of the epicuticle does not affect the rate of diffusion of the hypochlorite ion. In alkaline solutions the negatively charged subcuticle retards the penetration of anions, so that diffusion through this membrane determines the overall rate of reaction.

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REFERENCES

Alexander, P. (1950). *J. Soc. Dy. Col., Bradford*, **66**, 349.
Alexander, P., Carter, D. & Hudson, R. F. (1949). *J. Soc. Dy. Col., Bradford*, **65**, 152.
Alexander, P. & Earland, C. (1950). *Text. Res. J.* **20**, 298.
Alexander, P., Earland, C. & Happey, F. (1950). *Nature, Lond.*, **166**, 396.
Alexander, P., Gough, D. & Hudson, R. F. (1949). *Trans. Faraday Soc.* **45**, 1058, 1109.
Alexander, P. & Hudson, R. F. (1949). *J. phys. colloid Chem.* **53**, 733.
Alexander, P. & Hudson, R. F. (1950). *Text. Res. J.* **20**, 481.
Alexander, P., Hudson, R. F. & Fox, M. (1950). *Biochem. J.* **46**, 37.
Alexander, P. & Kitchener, J. A. (1950). *Text. Res. J.* **20**, 203.
Cuthbertson, W. R. & Phillips, H. (1945). *Biochem. J.* **39**, 7.
Kressman, T. R. E. & Kitchener, J. A. (1949). *Faraday Soc. Discussion*, no. 7, p. 90.
Kunin, R. & Myers, R. J. (1947). *J. phys. colloid Chem.* **51**, 1111.
Lindberg, J., Philip, B. & Gralén, N. (1948). *Nature, Lond.*, **162**, 458.
Mercer, E. H., Lindberg, J. & Philip, B. (1949). *Text. Res. J.* **19**, 678.
Rowen, J. W. & Blaine, R. L. (1947). *Industr. Engng Chem.* **39**, 1659.
Speakman, J. B. & Goodings, L. (1926). *J. Text. Inst., Manchr*, **17**, T 607.
Steinhardt, J. & Harris, M. (1940). *Bur. Stand. J. Res., Wash.*, **24**, 335.
Teorell, T. (1949). *Ann. Rev. Physiol.* **11**, 545.
Zahn, H. (1950). *Melliand Textilber.* **31**, 36.

The Biosynthesis of Choline Methyl Groups by the Rat

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It has been known for some time that the supply of choline or betaine to a methionine-free diet enables the white rat to use homocystine as the sole sulphur-containing amino-acid for growth purposes (du Vigneaud, Chandler, Moyer & Keppel, 1939). This effect has been shown to be due to the utilization of the methyl groups of choline for the biological synthesis of methionine from homocystine (Simmonds, Cohn, Chandler & du Vigneaud, 1943). The reverse reaction, i.e. the transfer of the methyl group of methionine to choline, also occurs (du Vigneaud, Cohn, Chandler, Schenk & Simmonds, 1941). The mechanism of the transfer of labile methyl groups has been elucidated by feeding methionine containing deuterium and ^{14}C in the methyl group. The choline methyl groups were found to contain both isotopic tracers in the same ratio as in the methionine fed, demonstrating the transfer of the methyl group as such (Keller, Rachele & du Vigneaud, 1949).

Recently it has been shown, by feeding methionine labelled with ^{14}C in the methyl group, that labile methyl groups are rapidly oxidized to carbon dioxide by the intact rat (Mackenzie, Chandler, Keller, Rachele, Cross & du Vigneaud, 1949). When choline, labelled with ^{14}C in the methyl groups, was administered to rats, the tracer was found in the β-position of serine (Sakami, 1949*a*) suggesting that formate is an intermediate in the oxidation of labile methyl groups, since the β-carbon atom of serine can arise by condensation of formate and glycine *in vivo* (Sakami, 1948).

It was therefore of some interest to investigate whether the formation of choline methyl groups from simple precursors by a reverse reaction of the above oxidation can occur. Sodium formate and methanol, labelled with ^{14}C, were chosen as two easily accessible one-carbon compounds whilst α-labelled glycine and β-labelled serine were considered to be likely physiological precursors of active one-carbon compounds. *In vitro* work with liver slices had shown that formate is produced from the α-carbon atom of glycine (Siekevitz, Winnick & Greenberg, 1949), and after completion of this work it was reported that both the α-carbon atom of glycine and the β-carbon atom of serine give rise to formaldehyde and formate (Siekevitz & Greenberg, 1950).

A preliminary communication was given by Arnstein (1950*a*).

EXPERIMENTAL

Animals and diet. Except in Exp. 1, where albino rats of Institute strain were used, all the animals were Wistar strain rats supplied by Dr K. Coward, Pharmaceutical Society.

The animals were kept on Institute stock diet of the following composition: wholemeal flour, 46%; Sussex ground oats, 40%; fish meal, 8%; dried skimmed milk, 3%; cod liver oil, 1%; dried yeast, 1%; NaCl, 1%.

The isotopically labelled compounds were fed overnight mixed with diet (10 g.), unless otherwise stated. In some cases sodium benzoate, *p*-aminobenzoic acid or γ-phenyl-α-aminobutyric acid were also fed in connexion with another investigation. After a time interval of between 1 and 5 days the animals were killed by exsanguination under anaesthesia. The details of the feeding experiments are given in Table 1.

Isolation of choline and creatine. After removal of the liver, the carcasses were minced and extracted with ethanol; choline was precipitated as the reineckate, converted to the chloroplatinate and degraded to trimethylamine chloroplatinate (du Vigneaud *et al.* 1941). In two experiments, creatinine was converted to creatine and isolated as the picrate of the potassium salt (du Vigneaud *et al.* 1941).

Synthesis of ^{14}C-choline and degradation of synthetic material. In order to check the assumption that the carbon atoms of trimethylamine originate only from the methyl groups of choline, the degradation was repeated on choline, labelled with ^{14}C in the hydroxyethyl group, which had been synthesized by the following series of reactions. It is probable that the last step, i.e. the condensation of 2-chloroethanol with trimethylamine, takes place via ethylene oxide, in which case the choline would contain radioactive isotope in both C

Table 1. *Details of administration of isotopically labelled compounds*

	Details of animals				Details of labelled compound given					
Exp. no.	No. of rats	Sex	Aggregate body wt. (g.)	No. of days kept alive after feeding tracer	Compound fed	Wt. (mg.)	Radioactivity (μc.)	Atom % excess	Specific radioactivity (μc./mmol.)	Quantity fed (mmol./100 g. body wt.)
1 (*a*)*	3	F	900	5	DL-$^{14}CH_2OH.CHNH_2.CO_2H$	240·2	6·0	—	2·62	0·254
2 (*b*)	2	F	550	2·5	L-$^{14}CH_2OH.CHNH_2.CO_2H$	122·0	10·0	—	8·60	0·211
3 (*c*)	2	F	508	2·5	D-$^{14}CH_2OH.CHNH_2.CO_2H$	142·6	11·7	—	8·59	0·267
4 (*d*)	1	F	302	3	$^{14}CH_2NH_2.CO_2H$	9·6	9·6	—	75·07	0·0423
5	2	F	545	2	$^{14}CH_2NH_2.CO_2H$†	39·8	39·8	—	75·07	0·0973
6 (*e*)	1	F	260	1	$^{14}CH_3OH$	13·7	10·3	—	24·09	0·1645
					$CH_2{}^{15}NH_2.CO_2H$	10·0	—	30·2	—	0·0513
7 (*f*)	1	M	387	3	$H^{14}CO_2Na$	41·7	22·3	—	36·38	0·158
					$CH_2{}^{15}NH_2.CO_2H$	10·0	—	30·2	—	0·0345
8 (*g*)	2	F	590	4	$CH_2NH_2.{}^{14}CO_2H$	45·6	20·5	—	15·61	0·223
					$CH_2{}^{15}NH_2.CO_2H$	53·0	—	30·2		

* Substances added to diet:

(*a*) 200 mg. *p*-aminobenzoic acid, 200 mg. γ-phenyl-α-aminobutyric acid/animal (2 rats); 200 mg. sodium benzoate, 200 mg. γ-phenyl-α-aminobutyric acid/animal (1 rat).

(*b*) 100 mg. sodium benzoate, 100 mg. γ-phenyl-α-aminobutyric acid/animal.

(*c*) 200 mg. sodium benzoate, 200 mg. γ-phenyl-α-aminobutyric acid/animal.

(*d*) 200 mg. *p*-aminobenzoic acid, 200 mg. γ-phenyl-α-aminobutyric acid.

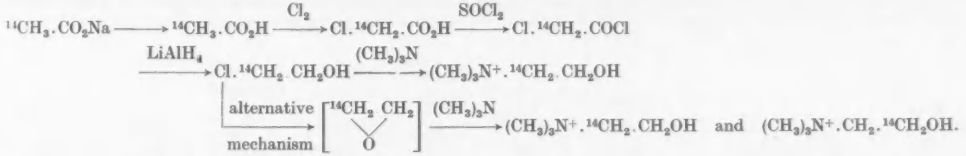
(*e*) 50 mg. sodium benzoate.

(*f*) 54 mg. sodium benzoate.

(*g*) 50 mg. sodium benzoate/animal; 50 mg. sodium benzoate/animal, 3 days later.

† By intraperitoneal injection.

atoms of the hydroxyethyl group, but this does not affect the conclusions drawn from the degradation experiment:



^{14}C-Methyl-labelled sodium acetate (5·7 mg., 69·5 μc.; obtained from the Radiochemical Centre, Amersham) was diluted with non-radioactive acetic acid (2·60 g., 43·4 mmol.) and converted to chloroacetic acid (Ostwald, 1948). The crude product was refluxed with $SOCl_2$ (5 ml.) for 5 hr. and left at room temperature for 2 days (McMaster & Ahmann, 1928). Pure chloroacetyl chloride (6 g., 53·2 mmol.) was then added as carrier and the product was distilled using a Vigreux column (6 × 1 cm.), the fraction boiling at 100–106°/760 mm. Hg being collected. It was found impossible to remove all the $SOCl_2$ by fractional distillation, but the material was sufficiently pure for the next step. A solution of lithium aluminium hydride (2·75 g.) in dry ether (50 ml.) was added slowly with stirring to a cooled solution of the acid chloride in dry ether (50 ml.) (Sroog, Chih, Short & Woodburn, 1949). When the reaction was completed, dil. H_2SO_4 was added and the aqueous layer was extracted several times with ether. The ether extract was evaporated and the residue was distilled *in vacuo*, using a Vigreux column (6 × 1 cm.), the fraction b.p. 80–90°/15 mm. Hg being collected (yield, 1·71 g.). 2-Chloroethanol (1·39 g.) was treated with trimethylamine (2 ml.) in a sealed tube. After 48 hr. at room temperature the tube was heated at 90–100° for 4 hr. (Wurtz, 1868). The excess trimethylamine was then allowed to evaporate, the last traces being removed by heating the product at 215° in a high vacuum. Weight of residue was 2·3 g. A small portion of the choline chloride was converted to the chloroplatinate, m.p. 240–245° (decomp.). Radioactivity: 591 ± 13·7 counts/min./mg. sample, counted as an infinite thickness sample of 1·2 sq. cm. area. The calculated specific activity of the choline (free base) was 0·204 μc./mmol. Degradation of this synthetic material with alkaline permanganate (du Vigneaud *et al.* 1941) afforded trimethylamine chloroplatinate which was completely non-radioactive.

Synthesis of labelled compounds used in feeding experiments

[β-^{14}C]-*Serine.* The synthesis of this compound has been described in a preliminary communication (Arnstein, 1949).

[α-^{14}C]-*Glycine.* [α-^{14}C]-Bromoacetic acid (supplied by the Radiochemical Centre, Amersham) was treated with excess NH_3 (sp.gr. 0·88), essentially as described by Olsen, Hemingway & Nier (1943).

[^{14}COOH]-*Glycine.* ^{14}C-KCN (supplied by the Radiochemical Centre, Amersham) was reacted with *N*-chloromethylphthalimide (Sakami, Evans & Gurin, 1947). The product was hydrolysed with HCl and the glycine isolated from aqueous ethanolic solution by addition of pyridine.

Sodium [^{14}C]-*formate.* Potassium [^{14}C]-formate was prepared by hydrolysis of aqueous K^{14}CN at 250° for 2 hr. (Grant & Turner, 1950) and converted to sodium formate after vacuum distillation of the formic acid.

[^{14}C]-*Methanol.* This was supplied by the Radiochemical Centre, Amersham; it was diluted about 40-fold before use to give material of a specific activity of about 25 μc./mmol.

RESULTS

The specific radioactivity of choline chloroplatinate, potassium creatine picrate and xanthydryl urea (when isolated) and the results of the degradation of choline to trimethylamine are given in Table 2. In order to compare different experiments the relative isotope coefficient (RIC) and the concentration coefficient (CC) were calculated (Table 3) as given by Anker (1948). The dilution factor is defined as the reciprocal of the concentration coefficient (CC) and gives the dilution of isotopic tracer which would

Table 2. *Specific radioactivities and* ^{15}N *contents of isolated compounds*

(The radioactivity was measured on a Geiger counter, using infinite thickness samples. The counting error is given as the standard deviation.)

Exp. no.	Labelled compound fed	Potassium creatine picrate μc. × 10^{-6}/mg.	Xanthydryl urea (μc. × 10^{-6} mg.)	Choline chloroplatinate μc. × 10^{-6} mg.	Choline chloroplatinate Atom % excess ^{15}N	Trimethylamine chloroplatinate (μc. × 10^{-6}/mg.)
1	DL-$^{14}CH_2OH.CHNH_2.CO_2H$	—	—	10·35 ± 0·2	—	1·68 ± 0·15
2	L-$^{14}CH_2OH.CHNH_2.CO_2H$	1·2 ± 0·6	12·5 ± 0·2	32·1 ± 0·5	—	7·85 ± 0·9
3	D-$^{14}CH_2OH.CHNH_2.CO_2H$	—	22·5 ± 1·5	Inactive	—	—
4	$^{14}CH_2NH_2.CO_2H$	—	14·45 ± 0·3	30·2 ± 1·3	—	6·47 ± 0·6
5	$^{14}CH_2NH_2.CO_2H$ (by intraperitoneal injection)	—	—	12·6 ± 0·6	—	0·67 ± 0·15
6	$^{14}CH_2OH$	—	12·55 ± 1·3	45·0 ± 1·0	—	20·9 ± 1·0
	$CH_2{}^{15}NH_2.CO_2H$	—	—	—	0·009	—
7	$H^{14}CO_2Na$	—	76·9 ± 1·8	73·5 ± 2·4	—	17·1 ± 0·8
	$CH_2{}^{15}NH_2.CO_2H$	—	—	—	0·006	—
8	CH_2NH_2 $^{14}CO_2H$	—	—	Inactive	—	—
	$CH_2{}^{15}NH_2.CO_2H$	—	—	—	0·012	—

Table 3. *Dilution of isotopic tracer in choline and trimethylamine after feeding various labelled precursors*

(The relative isotope concentration (RIC) and concentration coefficient (CC) were calculated according to Anker (1948). The dilution factor is the reciprocal of the concentration coefficient. The percentage isotope in trimethylamine is given by the ratio ($RIC_{(trimethylamine)}/RIC_{(choline)}$) × 100.)

Exp. no.	Compound fed	Choline		Trimethylamine		Isotope in trimethylamine (%)	Dilution factor	
		RIC (× 10^{-4})	CC (× 10^{-3})	RIC (× 10^{-5})	CC (× 10^{-4})		Choline	Trimethylamine
1	DL-[β-^{14}C]-Serine	12·18	4·80	16·93	6·67	13·9	208	1449
2	L-[β-^{14}C]-Serine	11·50	5·45	24·13	11·40	21·0	184	878
4	[α-^{14}C]-Glycine	1·24	2·93	2·27	5·36	18·2	341	1866
6	[^{14}C]-Methanol	5·75	3·50	22·92	13·90	39·8	286	719
	[^{15}N]-Glycine	2·98	5·81	—	—	—	172	—
7	Sodium [^{14}C]-formate	6·23	3·93	12·41	7·84	19·9	254	1278
	[^{15}N]-Glycine	1·99	5·76	—	—	—	174	—
8	[^{14}COOH]-Glycine	—	—	—	—	—	—	—
	[^{15}N]-Glycine	7·39	3·31	—	—	—	302	—

have been observed if exactly 1 mmol. of labelled compound had been fed per 100 g. body weight.

Methanol, sodium formate, glycine (labelled in the α-position or with ^{15}N) and L-serine (labelled in the β-position) were found to be precursors of choline. The dilution of tracer was somewhat lower with L-serine than with the other compounds. Degradation of the choline showed that methanol was converted to choline methyl groups with greater efficiency than were the α-carbon atom of glycine, the β-carbon atom of L-serine or sodium formate. After administration of [^{14}COOH]-glycine or D-[β-^{14}C]-serine the choline was completely non-radioactive. [^{15}N]-Glycine, fed at the same time as the carboxyl-labelled compound, was incorporated into choline to about the same extent as α-labelled glycine.

Recently, other workers have also shown that methanol (du Vigneaud & Verly, 1950) and formate (Welch & Sakami, 1950; Sakami, 1950) are converted to choline methyl groups, though full experimental details have not yet been published.

DISCUSSION

The nutritional interrelationships of choline and methionine have been satisfactorily explained by the ability of these substances to act as donors of labile methyl groups (for a summary dealing with the transfer of methyl groups in the body see du Vigneaud, 1948). Only a few other compounds such as betaine and dimethylthetin (du Vigneaud, Moyer & Chandler, 1948) have been found to supply labile methyl groups and thus to replace choline or methionine in nutrition. On the other hand, dimethylaminoethanol, though a precursor of choline and resembling it in preventing fatty livers and haemorrhagic kidneys in rats (du Vigneaud, Chandler, Simmonds, Moyer & Cohn, 1946), is not available for growth purposes, presumably because its methyl groups are not metabolically labile.

Although *in vivo* synthesis of choline takes place, the animal would appear to be unable to synthesize methyl groups at a rate sufficient for optimal growth. Alternatively, the rat may synthesize dimethylaminoethanol, but conversion of this compound to choline may require a preformed labile methyl group.

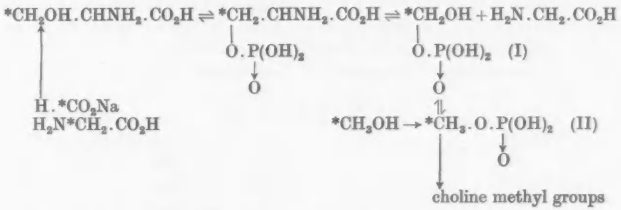
Hitherto there has been little experimental evidence to indicate whether biosynthesis of methyl groups occurs in the rat or whether all the methyl groups are derived from the diet. When the body water of the white rat, kept on a diet adequate in labile methyl groups, was enriched with heavy water, some deuterium was incorporated into the methyl groups of choline (du Vigneaud, Simmonds, Chandler & Cohn, 1945). Although this isotope might have been introduced into the methyl groups by an exchange of hydrogen atoms, e.g. by an oxidation-reduction mechanism, it was thought more likely that the white rat synthesized a small, but significant amount of methyl groups, even though preformed labile methyl groups were provided in the diet.

The present experiments show unequivocally that, on a stock diet containing an adequate amount of labile methyl groups, the white rat synthesized choline methyl groups from one-carbon precursors which can be derived from the α-carbon atom of glycine or the β-carbon atom of L-serine as well as from formate or methanol. D-[β-^{14}C]-Serine and [^{14}COOH]-glycine were not converted to choline. Since both these compounds are oxidized to carbon dioxide it is evident that the intact animal is unable to reduce carbon dioxide to methyl groups to any significant extent.

Earlier work with ^{15}N had shown that both ethanolamine and glycine are precursors of choline (Stetten, 1941), but it was not known whether ethanolamine arises by reduction of glycine, as suggested by Shemin (1946), or by decarboxylation of serine

(Stetten, 1942). Since glycine and serine are in dynamic equilibrium as shown by experiments with ^{15}N (Elliott & Neuberger, 1950), the mechanism of ethanolamine formation from these compounds can be better elucidated with isotopic carbon than with ^{15}N. L-[β-^{14}C]-Serine was found to be converted to choline with about the same efficiency as [^{15}N]-glycine, but [^{14}COOH]-glycine, as already mentioned, was not a precursor of choline. Since [^{14}COOH]-glycine is converted to serine containing tracer in the carboxyl group only (Sakami, 1948; Siekevitz *et al.* 1949), these experiments show that the ethanolamine moiety of choline, and hence presumably ethanolamine itself, is derived from serine with loss of the carboxyl group. In this connexion it is of interest that D-[β-^{14}C]-serine is not a precursor of either the ethanolamine moiety of choline or the methyl groups. This difference between D- and L-serine is one more illustration of metabolic differences between stereoisomers. Since L- but not D-serine is converted to glycine (Shemin, 1946), it may be presumed that the D-isomer does not split off the β-carbon atom as a one-carbon fragment and hence is not a precursor of methyl groups. On the other hand, both isomers are converted to acetate with about equal efficiency (Arnstein, 1950*b*).

The identity of the one-carbon methyl-group precursor is at present unknown. Although both formaldehyde and formate are degradation products of the β-carbon atom of L-serine and of the α-carbon atom of glycine (Siekevitz & Greenberg, 1950), these compounds may not be involved as such. It is possible that the precursor is a phosphorylated one-carbon compound derived from the β-carbon atom of serine during its conversion to glycine, thus: (*C = the labelled C atom)



According to this view, formate and the α-carbon atom of glycine would be converted to this one-carbon precursor via the β-carbon atom of serine (Sakami, 1948, 1949*b*), accounting for the greater dilution of isotopic tracer in the choline methyl groups after feeding these compounds (Table 3) compared with L-serine. The relatively efficient conversion of methanol to choline methyl groups can be explained by assuming its conversion to a precursor (II) which is metabolically closer to methyl groups than that derived from formate. By contrast, methanol and formate are converted to the ethanolamine moiety of choline with about equal efficiency, suggesting that both the compounds give rise to intermediates which condense with glycine to give serine and thence ethanolamine.

Since α-labelled glycine gives rise to serine containing approximately equal amounts of isotope in the α- and β-position (Sakami, 1949*b*), the ethanolamine derived from this precursor will be labelled in both carbon atoms. With the other compounds fed only the β-carbon atom of serine and hence only one carbon atom of ethanol amine will contain isotope. Allowing for this difference, the radioactivity per carbon atom in the methyl groups and in the ethanolamine moiety, obtained in the various experiments, has been calculated (Table 4). If the amount of labile methyl groups in the diet and their turnover

Table 4. *Calculated radioactivity of the methyl groups and ethanolamine moiety of choline derived from fed precursors*

Exp. no.	Compound fed	Specific radioactivity (μc./g.mol.) Choline	Trimethylamine	Ethanolamine moiety	Maximum no. of carbon atoms of choline which can be derived from labelled atom of precursor fed: Methyl groups	Ethanolamine moiety	Radioactivity/g.mol./C atom derived from precursor: Methyl group (*a*)	Ethanolamine moiety (*b*)	Ratio (*b*)/(*a*)
1	DL-[β-^{14}C]-Serine	3·19	0·444	2·75	3	1	0·148	2·75	18·6
2	L-[β-^{14}C]-Serine	9·89	2·08	7·81	3	1	0·693	7·81	11·4
4	[α-^{14}C]-Glycine	9·31	1·70	7·61	3	2	0·567	3·81	6·7
6	[^{14}C]-Methanol	13·86	5·52	8·34	3	1	1·84	8·34	4·5
7	Sodium [^{14}C]-formate	22·66	4·51	18·15	3	1	1·50	18·15	12·1

rate were known, the number of methyl groups synthesized from the fed precursors per molecule of choline could be calculated. As these factors were not ascertained no quantitative deductions are possible. It is of interest however that, compared with the ethanolamine formed, only a small number of methyl groups appear to have been synthesized by the animal from the fed precursors. It is possible that this apparently slow rate of methyl group synthesis is due mainly to the adequate supply of labile methyl groups in the diet.

SUMMARY

1. The biosynthesis of choline has been studied by feeding D- and L-[β-^{14}C]-serine, [α-^{14}C]-glycine, [^{14}COOH]-glycine, [^{14}C]-methanol and [^{14}C]-formate to adult rats.

2. It is concluded that L-serine is converted to the ethanolamine moiety of choline by loss of the carboxyl group. D-Serine is not a precursor of choline.

3. Methanol, formate, the β-carbon atom of L-serine and the α-carbon atom (but not the carboxyl carbon atom) of glycine are precursors of choline methyl groups.

4. Carbon dioxide, arising by *in vivo* oxidation of D-[β-^{14}C]-serine or [^{14}COOH]-glycine, is not converted to choline methyl groups.

5. The rate of synthesis of choline methyl groups from the above precursors appears to be slow compared with that of the ethanolamine moiety.

6. A synthesis of choline labelled with ^{14}C in the ethanolamine moiety is described.

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REFERENCES

Anker, H. S. (1948). *J. biol. Chem.* **176**, 1337.
Arnstein, H. R. V. (1949). *Nature, Lond.*, **164**, 361.
Arnstein, H. R. V. (1950*a*). *Biochem. J.* **47**, xviii.
Arnstein, H. R. V. (1950*b*). Unpublished observations.
Elliott, D. F. & Neuberger, A. (1950). *Biochem. J.* **46**, 207.
Grant, D. G. & Turner, H. S. (1950). *Nature, Lond.*, **165**, 153.
Keller, E. B., Rachele, J. R. & du Vigneaud, V. (1949). *J. biol. Chem.* **177**, 733.
Mackenzie, C. G., Chandler, J. P., Keller, E. B., Rachele, J. R., Cross, N. & du Vigneaud, V. (1949). *J. biol. Chem.* **180**, 99.
McMaster, L. & Ahmann, F. F. (1928). *J. Amer. chem. Soc.* **50**, 145.
Olsen, N. S., Hemingway, A. & Nier, A. O. (1943). *J. biol. Chem.* **148**, 611.
Ostwald, R. (1948). *J. biol. Chem.* **173**, 207.
Sakami, W. (1948). *J. biol. Chem.* **176**, 955.
Sakami, W. (1949*a*). *J. biol. Chem.* **179**, 495.
Sakami, W. (1949*b*). *J. biol. Chem.* **178**, 519.
Sakami, W. (1950). *Fed. Proc.* **9**, 222.
Sakami, W., Evans, W. E. & Gurin, S. (1947). *J. Amer. chem. Soc.* **69**, 1110.
Shemin, D. (1946). *J. biol. Chem.* **162**, 297.
Siekevitz, P. & Greenberg, D. M. (1950). *Fed. Proc.* **9**, 227.
Siekevitz, P., Winnick, T. & Greenberg, D. M. (1949). *Fed. Proc.* **8**, 250.
Simmonds, S., Cohn, M., Chandler, J. P. & du Vigneaud, V. (1943). *J. biol. Chem.* **149**, 519.
Sroog, C. E., Chih, C. M., Short, F. A. & Woodburn, H. M. (1949). *J. Amer. chem. Soc.* **71**, 1710.
Stetten, D. (1941). *J. biol. Chem.* **140**, 143.
Stetten, D. (1942). *J. biol. Chem.* **144**, 501.
du Vigneaud, V. (1948). *Proc. Amer. phil. Soc.* **92**, 127.
du Vigneaud, V., Chandler, J. P., Moyer, A. W. & Keppel, D. M. (1939). *J. biol. Chem.* **131**, 57.
du Vigneaud, V., Chandler, J. P., Simmonds, S., Moyer, A. W. & Cohn, M. (1946). *J. biol. Chem.* **164**, 603.
du Vigneaud, V., Cohn, M., Chandler, J. P., Schenk, J. R. & Simmonds, S. (1941). *J. biol. Chem.* **140**, 625.
du Vigneaud, V., Moyer, A. W. & Chandler, J. P. (1948). *J. biol. Chem.* **174**, 477.
du Vigneaud, V., Simmonds, S., Chandler, J. P. & Cohn, M. (1945). *J. biol. Chem.* **159**, 755.
du Vigneaud, V. & Verly, W. G. (1950). *J. Amer. chem. Soc.* **73**, 1049.
Welch, A. D. & Sakami, W. (1950). *Fed. Proc.* **9**, 245.
Wurtz, A. (1868). *Liebigs Ann.* Suppl. **6**, 117.

The Inhibition of Oxidative Phosphorylation

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Loomis & Lipmann (1948) showed that low concentrations of 2:4-dinitrophenol (DNP) reversibly uncouple the phosphorylation associated with the oxidation of glutamate. This supported the hypothesis that agents such as DNP, which prevent the use of the energy provided by respiration and glycolysis, do so by inhibiting the formation of high-energy phosphate bonds (Lardy & Elvehjem, 1945; McElroy, 1947). The acceleration of respiration and glycolysis of intact cells by low concentrations of DNP (see Peiss & Field, 1948, for references) might thus be explained in terms of an increased availability of orthophosphate and of adeninenucleotide acceptors which normally limit the rate of carbohydrate breakdown (cf. Meyerhof & Geliazkowa, 1947). Johnson (1941) has suggested that the Pasteur effect is a consequence of the greater efficiency of aerobic phosphorylation compared with that associated with glycolysis, a view which is consistent with the inhibition of the Pasteur effect by DNP (Dodds & Greville, 1934). Lynen (1941) has indeed shown that respiration reduces the amount of orthophosphate available for yeast fermentation.

Some interest is therefore attached to the action of other compounds known to accelerate respiration and/or aerobic glycolysis on oxidative phosphorylation. For example, phenosafranine in suitable concentration will increase the aerobic glycolysis of rat-brain and tumour slices without affecting the respiration. Dyestuffs such as thionine accelerate the respiration of many tissues without influencing the aerobic glycolysis (Dickens, 1936*a*, *b*), while 4:6-dinitro-*o*-cresol increases both respiration and aerobic glycolysis (Dickens, 1939). We have, therefore, investigated the action of a number of known inhibitors of the Pasteur effect, as well as some metabolites of DNP, on the coupling between oxidation and phosphorylation. Two hormones, thyroxine and insulin, which we thought might possibly influence oxidative phosphorylation, have also been tested.

The enzyme systems that we have used in this investigation were those contained in cyclophorase preparations and in mitochondria of rat and rabbit liver and kidney. Hogeboom, Schneider & Pallade (1948) and Kennedy & Lehninger (1948, 1949) have shown that most of the enzymes of the tricarboxylic acid cycle, and also those catalysing fatty acid oxidation, are localized in the mitochondria. We have examined the possibility that the activity of crude preparations of tissue particles such as the 'cyclophorase system' (Green, Loomis & Auerbach, 1948) resides in the mitochondria.

METHODS

Enzyme preparations. Washed kidney and liver particles ('cyclophorase') were prepared by the method of Green *et al.* (1948). Purer preparations of the active particles were obtained by isolating the mitochondria according to the methods described by Schneider (1948). These methods were applied with success to rabbit and rat liver and kidney. The necessity for neutralization during homogenization (Green *et al.* 1948) is not apparent when the procedure is carried out in sucrose. All preparations were carried out in a cold room at 2°, a Servall SS1–*a* high-speed centrifuge being used for the mitochondrial isolations.

Hexokinase was prepared from baker's yeast by two methods. A crude preparation was obtained by a single ethanol precipitation (van Heyningen, 1942) and stored as a dry powder *in vacuo* at 2°. For use, 100 mg. of the powder was rubbed up with 10 ml. ice-cold water and centrifuged; 0·2 ml. of the supernatant was used per flask. A purer preparation of the enzyme was obtained by the method of Berger, Slein, Colowick & Cori (1946) as described by Cross, Covo, Taggart & Green (1949); the preparation was stored as a frozen solution at $-10°$, and retained its activity for many months. The conditions for autolysis of baker's yeast described by Berger *et al.* (1946) were found to be unsatisfactory and those described by Bailey & Webb (1948) were used instead.

Estimations. Inorganic orthophosphate was determined either by the method of Fiske & Subbarow (1925) or that of Lowry & Lopez (1946). Glucose was determined by the method of Nelson (1944). O_2 uptakes were determined manometrically, using Warburg flasks equipped with one side arm; the gas phase was air.

For the estimation of the P/O ratio (atoms of inorganic orthophosphate P disappearing/atoms O taken up from free O_2), a fortified medium was used containing the following: 0·001 M-adenosine-5-phosphate (AMP); 0·0067 M-$MgSO_4$; 0·02 M-potassium orthophosphate, pH 7·4; 0·01 M-substrate; 0·026 M-fructose and 0·2 ml. hexokinase solution.

The flasks were kept ice cold and the enzyme preparation was added immediately it was prepared. NaF was then added to give a final concentration of 0·013 M. The final volume in the centre compartments of the flasks was 3·0 ml. When a 'cyclophorase' preparation was used, this was

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added as 1·0 ml. of suspension in 0·9% (w/v) KCl. Where mitochondria were used, they were added to the flasks suspended in 0·25M-sucrose (0·5 ml., corresponding to 250–500 mg. fresh tissue), and in this case cytochrome *c* and KCl were added to the flasks to give final concentrations of 0·00001 and 0·025M respectively.

Trichloroacetic acid (0·5 ml. of 87·5%, w/v) was placed in the side arm of each flask. Immediately all components had been added to the flasks they were placed in the bath at 25 or 38° and allowed to equilibrate for 5 or 10 min. Four flasks were used for each determination. Two were deproteinized at the end of the equilibration period by tipping in the trichloroacetic acid, while the O_2 uptake was determined in the second pair for given periods (usually about 10 min.). These were then deproteinized. The contents of all flasks were made to 5·0 ml. with water, the precipitates centrifuged off and the supernatants were then analysed for inorganic ortho-P. In addition, samples were frequently analysed for the presence of acid-labile P (hydrolysis for 10 min. at 100° in N-HCl) and more specifically for inorganic pyro-P by the method of Cohn & Kolthoff (1942), which was modified so that pyro-P could be determined spectrophotometrically after hydrolysis. All results quoted are the means of duplicate determinations. Hexokinase activity was determined in small centrifuge tubes, in the presence of veronal-acetate buffer, pH 7·4, under the conditions stated by Bailey & Webb (1948), by estimation of glucose consumption or of the change in easily hydrolysable P.

Adenosinetriphosphatase (ATP-ase) and inorganic pyrophosphatase were determined in small tubes under the conditions summarized in Table 2.

Reagents. All solutions were made up in glass-distilled water, and neutralized to pH 7 if necessary by the use of KOH or HCl. Adenosinetriphosphate (ATP) was obtained as the dibarium salt from Boots Pure Drug Co. Ltd. Since some preparations contained inhibitory substances they were purified by reprecipitation as the Hg and Ba salts by the method of Dounce, Rothstein, Beyer, Meier & Freer (1948). The purified product yielded the theoretical amount of orthophosphate after hydrolysis in N-HCl at 100° for 10 min. After incubation with a four-times precipitated myosin preparation (Bailey, 1942), 48% of the theoretical pyrophosphate-P appeared as ortho-P. Adenosine-5-phosphate was obtained from Light & Co. Ltd. Cozymase was prepared by the method of Williamson & Green (1940). Purity determined spectrophotometrically after enzymic reduction was approx. 50%. Octanoic acid was purified by redistillation *in vacuo*. 'Hexose monophosphate' (an equilibrium mixture of glucose-6-phosphate and fructose-6-phosphate) was prepared by the method of Dubois & Potter (1943). Aneurin pyrophosphate was obtained from Roche Products Ltd. Cytochrome *c* was prepared by the method of Keilin & Hartree (1937). Most of the dyestuffs were obtained from G. Gurr Ltd. We are indebted to Dr H. McIlwain for a sample of ethyl red presented to him by Dr F. Hamer; to Dr H. King, F.R.S., for a specimen of 1:11-diamidinoundecane and to Dr J. M. Barnes for specimens of nitrophenols. Thyroglobulin was prepared from sheep thyroid glands by the method of Rossiter (1940). L-Thyroxine was a gift from Sir Charles Harington, F.R.S. 4:4′-Di(*p*-chlorophenyl)-1:1:1-trichloroethane (DDT) was suspended by the method described by Judah (1949). In all cases where effectors were dissolved in solvents other than water, the appropriate controls were run. Veronal-acetate buffer was prepared by the method of Michaelis (1931).

RESULTS

Initial experiments were carried out by the method of Potter (1945, 1947), in which water homogenates are used as a source of enzyme. Though oxygen uptakes were in good agreement with those reported by Potter, no phosphate uptake was ever observed. The experimental procedure described by Potter was rigidly adhered to.

Goranson & Erulkar (1949) have recently reported experiments with this type of preparation in which phosphorylation was obtained. Their value of 0·3 for the P/O ratio with succinate as substrate is unacceptably low. In view of the fact that homogenization in water destroys the mitochondria (Hogeboom *et al.* 1948) these results are not surprising.

Preliminary experiments with 'cyclophorase' and mitochondria on the conditions necessary for obtaining optimum values for the P/O ratio are in substantial agreement with those of Cross *et al.* (1949), and need not be discussed further. Table 1 shows the values for the P/O ratio obtained with different substrates and preparations at 25 and 38°. One finding justifies comment. It will be seen that with octanoate as substrate a clear-cut phosphorylation was observed, the phosphate uptake being many times the value expected from the oxidation of succinate added to prime the reaction. The latter experiments were conducted in the absence of fluoride which completely abolishes fatty acid oxidation at the concentrations used and which decreases glutamate oxidation by some 50% at 25°. It can be seen that in the absence of fluoride the P/O ratio with glutamate as substrate is markedly reduced. In many of these experiments, analyses for inorganic pyro-P were carried out. In no case was an accumulation of this compound observed. On a few occasions, inorganic pyro-P (10 μmol.) was added to flasks, and, upon estimation at the end of the experiment, no change in concentration was found. These observations clearly do not rule out the possibility that the compound was undergoing exchange with inorganic ortho-P in the suspension medium.

Since the results obtained with 'cyclophorase' could be duplicated with mitochondria, it was reasonable to suppose that the latter were the active agents in the 'cyclophorase' system. This was confirmed by resuspending the centrifuged particles in 0·88M-sucrose and subjecting them to differential centrifugation. The ability to carry out oxidative phosphorylation was recovered quantitatively in the mitochondrial fraction, the cytological characteristics of which were confirmed by phase-contrast microscopy and differential staining.

Some authors have corrected the observed P/O ratio for loss of esterified phosphorus by ATP-ase

Table 1. P/O *ratios with different substrates and preparations*

(P/O ratio = atoms of inorganic orthophosphate P disappearing/atoms O taken up from free O_2. The systems used are described in the text. 30 μmol. of substrate added in each case, except for succinate (100 μmol.) and octanoate (6·0 μmol.)

Substrate	Enzyme preparation	Temp. (°)	O_2 uptake (μg. atoms)	Inorganic ortho-phosphate (μg. atoms)	P/O ratio	Duration of experiment (min.)
Glutamate	Rabbit kidney 'cyclophorase'	25	8·85	15·9	1·8	10
Glutamate	Rabbit kidney 'cyclophorase'	25	4·53	11·3	2·5	5
Glutamate	Rat liver 'cyclophorase'	25	6·15	10·7	1·75	6
Glutamate	Rat liver 'cyclophorase'	25	5·35	13·5	2·52	7
Glutamate	Rat liver mitochondria	38	8·2	17·2	2·1	20
Glutamate	Rat liver mitochondria	38	21	22·2	1·05*	10
Glutamate	Rat liver mitochondria (Same preparation)	38	16·8	30·4	1·81	10
Succinate	Rabbit kidney 'cyclophorase'	38	33·2	29·4	0·88	8
β-Hydroxybutyrate	Rabbit kidney 'cyclophorase'	38	7·4	8·05	1·09	30
β-Hydroxybutyrate	Rabbit kidney mitochondria	38	15·1	15·1	1·0	43
β-Hydroxybutyrate	Rat liver mitochondria	38	17·8	24·0	1·35	8
Octanoate + 1 μmol. succinate	Rat liver mitochondria	38	44·5	25·0	0·56*	20
Pyruvate	Rat liver mitochondria	25	7·0	16·8	2·4	10

* No NaF added.

Table 2. *ATP-ase and inorganic pyrophosphatase of particles*

(The enzyme suspension (0·5 ml.) was added to a medium consisting of 0·017 M-veronal-HCl buffer, pH 7·4; 0·0067 M-$MgSO_4$; ATP (equivalent to 140 μg. easily hydrolysable P), or $Na_4P_2O_7$ (equivalent to 120 μg. pyro-P) and NaF as indicated. Final volume 1·2 ml. Duration of experiment 10 min.)

Enzyme preparation	Temp. (°)	Substrate	Hydrolysis (%) +0·02 M-NaF	Hydrolysis (%) No NaF
Rat liver 'cyclophorase'	25	ATP	44	99·5
Rat liver 'cyclophorase'	25	$Na_4P_2O_7$	8·0	86·0
Rabbit kidney 'cyclophorase'	25	ATP	33·0	65·0

action in the enzyme preparation. Ochoa (1943) has published evidence that the ATP-ase activity of heart homogenates is not completely inhibited by 0·02 M-fluoride. Ogston & Smithies (1948) have objected to the use of such corrected P/O ratios and have suggested that the sample of ATP used by Ochoa was heavily contaminated with inorganic pyrophosphate. It was further suggested by these authors that ATP-ase was completely inhibited, while the inorganic pyrophosphatase was unaffected by the concentrations of fluoride used.

We have investigated the influence of 0·02 M-sodium fluoride on the ATP-ase and inorganic pyrophosphatase activity of 'cyclophorase' isolated from liver and kidney. The ATP used was shown by enzymic dephosphorylation with four-times precipitated myosin to be practically free of inorganic pyrophosphate. Table 2 summarizes the results of these experiments, which demonstrate that the ATP-ase of liver and kidney is partially inhibited by 0·02 M-fluoride, while the inorganic pyrophosphatase is more powerfully inhibited.

Other experiments showed that the dephosphorylation of hexose mono- and di-phosphate was not marked in cyclophorase, and that 0·02 M-fluoride depressed this appreciably.

The P/O ratios recorded in this paper are not corrected for phosphatase action.

Inhibitors of oxidative phosphorylation

Unless otherwise stated, all experiments on the depression of oxidative phosphorylation were carried out at 25° with glutamate as substrate. Initial experiments confirmed the observation of Loomis & Lipmann (1948) that low concentrations of DNP powerfully inhibit the rate of phosphorus uptake while leaving respiration practically unaltered. Typical experiments are recorded in Table 3, which also shows that a similar effect was obtained with succinate and β-hydroxybutyrate as substrates, though in the case of these some inhibition of respiration was observed. A number of related nitrophenols were tested in this system. Table 4 shows that whereas 4-amino-2-nitrophenol and 2-amino-4-nitrophenol, known metabolites of 2:4-dinitrophenol (Guerbet & Meyer, 1932) did not affect the P/O ratio at the concentrations tested, 2:4-dinitro-1-naphthol-7-sulphonic acid and picramic acid were inhibitory, but not to such an extent as DNP.

Table 5 summarizes the results of experiments on the influence of various Pasteur inhibitors and related compounds on the P/O ratio. A number of

Table 3. *Depression of* P/O *ratio by DNP*

(Systems as described in text and Table 1.)

Substrate	Enzyme preparation	Concn. of DNP $\times 10^{-4}$ M	Temp. (°)	O_2 uptake (μg. atoms)	Inorganic ortho-phosphate uptake (μg. atoms)	P/O ratio
Glutamate	Rabbit kidney 'cyclophorase'	Nil	25	4·53	11·3	2·5
		1·0		4·55	0·0	—
Glutamate	Rabbit kidney 'cyclophorase'	Nil	25	3·54	6·15	1·74
		0·1		3·84	1·55	0·405
Succinate	Rabbit kidney 'cyclophorase'	Nil	38	33·2	29·4	0·88
		0·5		24·1	1·1	0·045
β-Hydroxybutyrate	Rabbit kidney 'cyclophorase'	Nil	38	7·4	8·05	1·09
		1·0		4·15	0·0	—
β-Hydroxybutyrate	Rat liver mitochondria	Nil	38	17·8	24·0	1·35
		1·0		13·0	0·0	—

Table 4. *Action of certain nitrophenols on* P/O *ratio*

(Rabbit kidney 'cyclophorase' used in every case, with glutamate as substrate.)

Compound	Concn. $\times 10^{-4}$ M	Percentage increase (+) or decrease (−) of O_2 uptake	Decrease of P uptake (%)	Depression of P/O ratio (%)
Picramic acid	1·0	−4	100	100
Picramic acid	2·0	−2	86	85·5
2:4-Dinitro-1-naphthol-7-sulphonic acid	1·0	+5·8	25	29·0
2:4-Dinitro-1-naphthol-7-sulphonic acid	2·0	−8·0	50	45·0
4-Amino-2-nitrophenol	2·0	−4	0·0	0·0
2-Amino-4-nitrophenol	2·0	+5·0	0·0	4·0
2:5-Dinitrophenol	1·0	−6·0	6·0	0·0

dyestuffs were found to be powerful inhibitors of phosphorus esterification at concentrations at which oxygen uptake was unaffected, namely, thionine, methylene blue, brilliant cresyl blue, pyocyanine, janus green B and phenosafranine. With the exception of the last, the results were reproducible. Using 'cyclophorase', concentrations of phenosafranine varying from 1 to 3×10^{-5} M inhibited the phosphate uptake of some preparations, while leaving that of others practically unaffected. Experiments with higher concentrations of the dyestuff were complicated by inhibitions of the oxygen uptake (50% inhibition at 10^{-4} M-phenosafranine with glutamate as substrate). McIlwain (1949) has shown that the breakdown of cozymase by rat-brain preparations is powerfully inhibited by low concentrations of phenosafranine. Although the enzyme system responsible for the breakdown of cozymase by rat brain is apparently quite different from that of rabbit kidney (McIlwain & Rodnight, 1949; Kornberg & Lindberg, 1948), it was considered that phenosafranine action might be related to the breakdown of cozymase by the 'cyclophorase'. The influence of the dye on the P/O ratio of a system, in which cozymase was added as a source of adenine nucleotide, was therefore investigated. It was found that 3×10^{-5} M-phenosafranine did not affect the replacement of adenosine-5-phosphate by cozymase and inhibited the P/O ratio to the same extent (40%) in the presence of either nucleotide. A possible reason for the inconsistent results with 'cyclophorase' may be that phenosafranine is strongly adsorbed by the preparations. More consistent inhibitions of the P/O ratio were obtained with purified mitochondrial preparations (Table 5), and this might be expected on the basis that 'cyclophorase' contains much debris which could effectively remove the dye from solution.

Of the other substances tested, ethyl red, guanidine, methyl guanidine, dimethyl guanidine, ethyl urethane and DDT affected neither respiration nor phosphate uptake in the concentrations used, 1:11-diamidinoundecane inhibited phosphate uptake at concentrations which did not depress the respiration. It is evident from the data included in Table 5 that the depression of the P/O ratio is not a simple function of the oxidation-reduction potential of the dyestuffs. Table 6 shows that neither thyroxine nor thyroglobulin when added *in vitro* affected the respiration or phosphate uptake. Similarly, insulin was devoid of influence on the P/O ratio, and did not affect the inhibition by DNP.

Hexokinase inhibition. It is evident that a lowering of the P/O ratio in the systems used in this in-

Table 5. *Depression of P/O ratio by Pasteur effect inhibitors and related compounds*

(Substrate glutamate throughout.)

Compound	Concn. $\times 10^{-4}$ M	Increase (+) or decrease (−) in O_2 uptake (%)	Decrease (−) or increase (+) in P uptake (%)	Depression of P/O ratio (%)	Oxidation/ reduction potential E'_0 at pH 7	Enzyme preparation
Ethyl red	0·1	+20	0·0	− 12	?	Rabbit kidney 'cyclophorase'
	0·2	0·0	− 9·0	− 9·0	—	Rabbit kidney 'cyclophorase'
Phenosafranine	0·1	+10	− 90·0	− 89·0	−0·252	Rabbit kidney 'cyclophorase'
	0·1	− 2·0	− 45·0	− 43·0	—	Rabbit kidney 'cyclophorase'
	0·3	0·0	− 6·0	− 6·0	—	Rabbit kidney 'cyclophorase'
	0·3	− 3·0	− 42·0	− 40·0	—	Rabbit kidney 'cyclophorase'
	0·3	−14·0	− 42·5	− 36·0	—	Rat liver mitochondria
	0·3	0·0	− 40·0	− 40·0	—	Rat liver mitochondria
	0·3	0·0	− 43·0	− 43·0	—	Rat liver mitochondria
Janus green	0·2	0·0	−100·0	−100·0	−0·256	Rabbit kidney 'cyclophorase'
	0·2	0·0	− 60·0	− 60·0	—	Rabbit kidney 'cyclophorase'
	0·2	−33·0	− 79·0	− 68·5	—	Rat liver mitochondria
	0·2	−29·0	− 72·0	− 61·0	—	Rat liver mitochondria
Guanidine-HCl	10·0	+18	+ 57·5	+ 30	—	Rabbit kidney 'cyclophorase'
	20·0	+44	+ 5	− 29	—	Rabbit kidney 'cyclophorase'
Methyl guanidine	9·1	− 3	− 30	− 27	—	Rabbit kidney 'cyclophorase'
	45	− 6	+ 35	+ 11	—	Rabbit kidney 'cyclophorase'
Dimethyl guanidine	8·0	− 3	− 19	− 13·5	—	Rabbit kidney 'cyclophorase'
	16	+12	− 10	− 20	—	Rabbit kidney 'cyclophorase'
Pyocyanine	4	+15	− 77	− 80	−0·034	Rabbit kidney 'cyclophorase'
	4	+ 3·8	− 78	− 79	—	Rabbit kidney 'cyclophorase'
	4	+ 2·0	− 46	− 44	—	Rabbit kidney 'cyclophorase'
1:11-Diamidinoundecane	1·0	+12·0	− 25·0	− 34·0	—	Rabbit kidney 'cyclophorase'
	1·0	−23·0	− 44·0	− 30·0	—	Rabbit kidney 'cyclophorase'
Methylene blue	0·2	0·0	− 11·0	− 11·0	0·011	Rabbit kidney 'cyclophorase'
	0·4	+13·0	− 31·0	− 39·0	—	Rabbit kidney 'cyclophorase'
Thionine	0·2	+17·0	− 50·0	− 58·0	0·062	Rabbit kidney 'cyclophorase'
	0·2	+ 9·7	− 54·0	− 58·0	—	Rabbit kidney 'cyclophorase'
	0·2	+10·8	− 44·0	− 48·0	—	Rabbit kidney 'cyclophorase'
	0·2	+ 5·0	− 61·5	− 63·0	—	Rabbit kidney 'cyclophorase'
Brilliant cresyl blue	0·5	+18·5	− 40·0	− 50·0	0·045	Rabbit kidney 'cyclophorase'
	0·4	− 2·0	− 40·0	− 41·0	—	Rabbit kidney 'cyclophorase'
Neutral red	1·4	+14·6	− 16·0	− 19·0	−0·340	Rabbit kidney 'cyclophorase'
Toluylene blue	1·4	+14·4	− 16·0	− 17·0	0·115	Rabbit kidney 'cyclophorase'

vestigation could be the result of an inhibition of the yeast hexokinase which is added, rather than a true uncoupling of phosphorylation from oxidation, or it might be a combination of both. All substances found to be inhibitors of the P/O ratio were therefore tested for their effect on the hexokinase preparation used. In no case was any significant inhibition observed.

Inhibition of fatty acid oxidation. Cross *et al.* (1949) have shown that low concentrations of DNP were powerful inhibitors of fatty acid oxidation by 'cyclophorase', and suggested that the inhibition was due to the lack of high energy phosphate bonds required to prime and maintain the reaction rather than an inhibition of the fatty acid oxidases. Thus it seemed likely that inhibitors of oxidative phosphorylation would also inhibit the oxidation of fatty acids. The substances were tested in a system with acetate as substrate and glutamate as primer, and the results of the experiments are summarized in Table 7. The inhibitory activity towards acetate oxidation was generally of the same order as the depression of the P/O ratio. The oxidation of acetate by 'cyclophorase' was found to be sensitive to the presence of fluoride, but it was not completely abolished as was octanoate oxidation. It can be seen from Table 7 that in the presence of 0·0067 M-fluoride, the inhibition of acetate oxidation by DNP is reduced some 40 %.

In several experiments the inhibitors were tipped in from the side arms of the flasks after measuring the oxygen uptake for 20 min. (after which time the oxidation of the primer was nearly complete). It was found that whereas DNP and other nitrophenols

Table 6. *Action of insulin, L-thyroxine and thyroglobulin on* P/O *ratio*

('Cyclophorase' used in both experiments.)

System	P/O ratio	Percentage depression of P/O ratio
Control	1·8	—
+Insulin*	1·66	7·5
$+10^{-4}$ M-DNP	0·18	90·0
$+10^{-4}$ M-DNP + insulin	0·116	96·0
Control	1·46	—
+L-Thyroxine $2·3 \times 10^{-4}$ M	1·32	10·0
+Thyroglobulin 3·9 mg./ml.	1·30	11·0

* Insulin (Burroughs-Wellcome Ltd.) was added as follows: 8·5 mg. was dissolved in 0·75 ml. 0·003 M-HCl and made up to 15·0 ml. with 0·1 M-phosphate buffer (pH 7·4). 0·15 ml. of this solution was added where indicated and the appropriate controls were run.

Mr J. H. Ottaway of the Department of Biochemistry, University College, London, has kindly informed us that this specimen of insulin was effective in enhancing the glucose uptake and glycogen synthesis of isolated rat diaphragms. The control system was identical with that described in the text and Table 1.

found to be true only in the cases where fluoride had been added to the reaction mixture. It can be seen from the results in Table 8 that in the absence of fluoride, 10^{-4} M-DNP did not stimulate the respiration of phosphate-deficient systems to the same extent as inorganic ortho-P, the absence of fluoride permitting a much more rapid respiration in the control flasks. The oxidation of glutamate in the absence as in the presence of fluoride was unaffected by DNP if phosphate were present in the suspension medium.

Many of the compounds tested as depressors of the P/O ratio were also examined for their ability to stimulate the respiration of phosphate-deficient systems. The results of the experiments are summarized in Table 8. It will be seen that picramic acid, methylene blue, brilliant cresyl blue and arsenate produced substantial acceleration of respiration, while phenosafranine, janus green, pyocyanine and 2:4-dinitro-1-naphthol-7-sulphonic acid had much less effect. Ethyl red was found to have no action on glutamate oxidation in phosphate-deficient systems.

Table 7. *Inhibition of acetate oxidation by* P/O *inhibitors*

(Rabbit kidney 'cyclophorase' used throughout. 30 μmol. of acetate as substrate, primed with 1 μmol. of glutamate. O_2 uptakes corrected for oxidation of the latter. In addition, the flasks contained 0·001 M-AMP, 0·0067 M-$MgSO_4$, 0·02 M-phosphate, pH 7·4 and inhibitors as indicated.)

Compound	Concentration	O_2 uptake (μl.)	Inhibition of O_2 uptake (%)	Depression of P/O ratio (%)
Nil	—	283	—	—
Ethyl red	2×10^{-5}	262	8·0	<20
1:11-Diamidinoundecane	10^{-4}	156	45·0	35
Picramic acid	2×10^{-4}	0·0	100·0	90·0
2:4-Dinitro-1-naphthol-7-sulphonic acid	2×10^{-4}	104	63·0	45·0
Nil	—	215	—	—
Phenosafranine	3×10^{-5}	124	43·0	40·0
Nil	—	300	—	—
DNP	10^{-4}	0·0	100·0	98·0
Nil	—	248	—	—
Thionine	2×10^{-5}	190	40·0	60·0
Nil + NaF, 0·0067 M	—	250	—	—
DNP + NaF, 0·0067 M	10^{-4}	139	56·0	—

cause an immediate inhibition of acetate oxidation, the inhibition by phenosafranine and janus green is small in the first 5 min., and increases rapidly with time.

Stimulation of the oxygen uptake of phosphate deficient systems

The oxidation of glutamate by either 'cyclophorase' or mitochondria was found to be much lower in systems to which inorganic ortho-P had not been added, the rate being some 35 % of the maximal. In confirmation of the observation of Loomis & Lipmann (1948), it was found that DNP stimulated the respiration of phosphate deficient systems to about the same extent as ortho-P itself. This was

The oxidation of succinate, however, is but slightly affected by the omission of phosphate from the medium, the rate being 70 % of the maximal. From Table 8 it can be seen that a small enhancement of the oxidation of succinate by phosphate deficient systems was observed in the presence of DNP. The interpretation of such experiments is complicated by the fact that DNP often caused inhibition of succinate oxidation. The oxidation of acetate was also found to be dependent on the presence of inorganic ortho-P. Table 8 shows that when this component was omitted from the suspension medium, acetate oxidation was practically abolished and neither DNP nor arsenate stimulated the oxidation.

Table 8. *Stimulation of O_2 uptake in P-deficient systems by P/O inhibitors*

(Rabbit kidney 'cyclophorase' used throughout; except where indicated, the systems were as described in Table 7. Where P was omitted, KCl was added to the same final molar concentration.)

Compound	NaF	Substrate	O_2 uptake (μl.)		Acceleration in absence of P by compound (%)
			P present	No P	
Nil	Nil	Acetate	162·0	1·0	—
DNP, 10^{-4}M	Nil	Acetate	0·0	0·0	—
Arsenate, 0·01 M	Nil	Acetate	0·0	0·0	—
Nil	0·013M	Glutamate	255·0	80·5	—
DNP, 10^{-4}M	0·013M	Glutamate	258·0	228·0	184
Nil	0·013M	Glutamate	197·0	59·0	—
Nil	Nil	Glutamate	296·0	118·0	—
DNP, 10^{-4}M	0·013M	Glutamate	197·0	195·0	203
DNP, 10^{-4}M	Nil	Glutamate	295·0	210·0	79
Nil	0·013M	Glutamate	180·0	65·0	—
Picramic acid, 2×10^{-4}M	0·013M	Glutamate	—	167·0	157
2:4-Dinitro-1-naphthol-7-sulphonic acid, 2×10^{-4}M	0·013M	Glutamate	—	110·0	70
Phenosafranine, 3×10^{-5}M	0·013M	Glutamate	—	80·0	23
Janus green, 2×10^{-5}M	0·013M	Glutamate	—	78·0	20
Ethyl red, 2×10^{-5}M	0·013M	Glutamate	—	65·0	0·0
Pyocyanine, 4×10^{-4}M	0·013M	Glutamate	—	91·0	40·0
Arsenate, 10^{-2}M	0·013M	Glutamate	—	181·0	179·0
Nil	0·013M	Glutamate	90·0	30·0	—
Methylene blue, 8×10^{-5}M	0·013M	Glutamate	—	81·0	170·0
Brilliant cresyl blue, 8×10^{-5}M	0·013M	Glutamate	—	77·0	156·0
Nil	0·013M	Succinate	237·0	168·0	—
DNP, 2×10^{-5}M	0·013M	Succinate	218·0	205·0	22·0

Stimulation of systems deficient in adenine nucleotide

The oxidation of glutamate by 'cyclophorase' or mitochondria to which inorganic orthophosphate but not a source of adenine nucleotide had been added was depressed to about the same extent as that of phosphate-deficient systems. It was found that DNP will accelerate the oxidation of glutamate in such systems, although the 'replacement' of adenine nucleotide is never complete. Fluoride affected the oxidation of glutamate under such conditions in the same manner as in phosphate-deficient systems, and the same was true of the enhancement of their respiration by DNP. The experiments summarized in Table 9 show that brilliant cresyl blue and methylene blue also increased the respiration of adenine nucleotide-deficient systems. Phenosafranine, however, did not affect glutamate oxidation under these conditions.

If inorganic phosphate and adenine nucleotide are together omitted from the reaction mixture, the stimulation of glutamate oxidation by DNP is maximal.

The procedures normally used for the isolation of either 'cyclophorase' or mitochondria yield suspensions which, despite repeated washing, contain appreciable amounts of adenine nucleotide and substances which appear as inorganic ortho-P either by the method of Fiske & Subbarow (1925) or by that of Lowry & Lopez (1946). The following technique yielded a suspension apparently free of adenine nucleotide.

Table 9. *Stimulation of O_2 uptake in AMP-deficient systems*

(Rabbit kidney 'cyclophorase' used throughout The 'complete system' was identical with that described in Table 7, except that glutamate (30 μmol.) was substrate.)

Components	O_2 uptake (μl.)	Acceleration by compound in absence of AMP (%)
Complete system	255·0	—
No AMP	99·0	—
No AMP + 0·0005 M-cozymase	183·0	85·0
No AMP + 10^{-4}M-DNP	179·0	81·0
No AMP, no P + 10^{-4}M-DNP	254·0	157·0
Complete system	197·0	—
No NaF	296·0	—
No AMP	79·0	—
No AMP, no NaF	103·0	—
No AMP + 10^{-4}M-DNP	131·0	66·0
No AMP, no NaF + 10^{-4}M-DNP	128·0	24·0
Complete system	213·0	—
No AMP	82·0	—
No AMP + 8×10^{-5}M-methylene blue	155·0	89·0
No AMP + 8×10^{-5}M-brilliant cresyl blue	138·0	69·0

Two rat livers (15 g.) were homogenized in a Waring blender in 0·25 M-sucrose at 0°. Nuclei and other debris were removed by centrifugation at 600 *g*

and the mitochondria were sedimented at 9000 *g* (15 min.). The particles were suspended in 0·9% (w/v) potassium chloride, containing 0·02 M-veronal-hydrochloric acid buffer (pH 7·7). They were subjected to four washings with this medium. The pellets resulting from the final centrifugation were then resuspended in 0·25 M-sucrose and sedimented at 9000 *g*, and then finally suspended in 15 ml. 0·25 M-sucrose. The suspension still contained material appearing as inorganic ortho-P (equivalent to 20 μg./ml.) by the method of Lowry & Lopez (1946). The suspension (0·5 ml.) was added to Warburg flasks and supplemented as usual in the case of the controls.

Table 10 shows that when adenosine-5-phosphate was omitted, the oxygen uptake was only 10% that of the complete system and the addition of 10^{-4} M-DNP failed to stimulate the respiration. In a phosphate-deficient system with added adenine nucleotide, however, the 'replacement' was maximal. Thus it appears that the 'replacement' of adenine nucleotide by DNP is dependent on the presence of a certain minimal amount of the former.

Table 10. *Failure of DNP to stimulate AMP-deficient systems*

(See text for description of method. Complete system as in Table 7.)

	O_2 uptake (μl.)/30 min.		
Compound added	Complete system	No P	No AMP
Nil	300	137	26
10^{-4} M-DNP	—	282	33

An attempt to remove all inorganic ortho-P was also made. The technique was similar to that used above, but 0·25 M-sucrose containing 0·02 M-veronal buffer (pH 7·7) was substituted for the potassium chloride solution. The particle preparation was indeed found to be free of ortho-P, but upon incubation in the Warburg flasks, the respiration fell off rapidly after 10 min. It was found on analysis that a considerable amount of ortho-P had been liberated from the mitochondria (approx. 100 μg. P), which had obviously been severely injured in some way.

DISCUSSION

It is impossible to over-emphasize the complexity of response of intact cells to the *in vitro* addition of the agents we have tested. The action of some of the latter varies from tissue to tissue and from species to species. The ionic composition of the suspension medium and the substrate employed also influence the results. Thus guanidine is an inhibitor of the Pasteur effect in brain slices, but does not have this effect in a number of other tissues (Dickens, 1939), while pyocyanine inhibits the aerobic glycolysis of a number of tumours (Friedheim, 1934; Dickens, 1936*a*), but accelerates that of brain slices (Young, 1937). Again, the aerobic glycolysis of rat kidney is unaffected by 6×10^{-5} M-thionine in the presence of glucose, but is considerably increased when lactate is present. The latter substance itself evokes a considerable aerobic glycolysis (Dickens, 1936*a*).

Seits & Engelhardt (1949), from a study of DNP, azide, arsenate, ethyl carbylamine and sodium nitrite on respiration, glycolysis and phosphorylation in yeasts, erythrocytes and tumours, have concluded that agents abolishing the Pasteur effect are, in general, inhibitors of respiratory phosphorylation. Many of the compounds listed in this paper as inhibitors of oxidative phosphorylation are indeed Pasteur-effect inhibitors in certain tissues, but it seems clear that, in view of the findings with intact cells, no comparison should be drawn between their activities in a cell-free system and on tissue slices. In addition, the question of tissue and species specificity remains an obstacle to generalization.

The action of the dyestuffs methylene blue, brilliant cresyl blue and phenosafranine merits further attention. The first two accelerate the respiration of slices, and it is often assumed that they do so by acting as supplementary electron carriers in respiratory systems (Harrop & Barron, 1928). The possibility must now be raised that their action is due to a depression of aerobic phosphorylation in the cells concerned.

With regard to phenosafranine, McIlwain (1949) has suggested that its action may be associated with its ability to inhibit the enzymic degradation of cozymase which was described by him. It should be pointed out that at least two mechanisms of cozymase breakdown exist (Kornberg & Lindberg, 1948; Kornberg & Pricer, 1950), and it is not yet known whether Kornberg's nucleotide pyrophosphatase is inhibited by phenosafranine. Furthermore, it is not certain that either of these degradative enzymes will act on coenzyme tightly bound to particles as they will when the substance is free in solution.

Our finding that the *in vitro* addition of thyroxine and thyroglobulin does not affect the P/O ratio strongly suggests that the thyroid hormone does not accelerate metabolic processes by interfering with oxidative phosphorylation in a manner similar to DNP.

We were unable to show any effect of insulin on oxidative phosphorylation. Recently, Polis, Polis, Kerrigan & Jedeikin (1949) have reported that insulin often increases the P uptake coupled with α-ketoglutarate oxidation by 'cyclophorase'. In order to demonstrate this effect they either employed systems with suboptimal amounts of adenosine-5-phosphate or added a magnesium-activated ATP-ase. In either case, the P/O ratios they obtained were far from maximal. We have not attempted to corro-

borate these experiments. It is of some interest that insulin did not reverse the inhibitory action of DNP.

In considering the apparent 'replacement' of inorganic ortho-P by DNP and other agents in systems which are deficient in ortho-P, the problem of the physical state of the particles must arise. Slater (1949) has demonstrated that the heart and kidney succinoxidase preparations used by him are sensitive to the phosphate concentration of the medium. He has found that substances such as denatured globin will accelerate the oxygen uptake of systems which are deficient in inorganic ortho-P, and suggests that phosphate has an effect on the structure of the particles composing the enzyme system. It seems impossible to rule out the operation of such factors in experiments with mitochondria or 'cyclophorase' suspended in similar media. Slater's results serve to emphasize the caution required in the explanation of results obtained from such complex systems as have been considered.

Our demonstration of the stimulation of respiration in the adenine nucleotide-deficient system by DNP, on the other hand, is not easily explained in the same way. The fact that DNP cannot enhance the respiration of adenine nucleotide-deficient systems except in the presence of a certain small amount of the latter is of interest. It might imply that adenosine-5-phosphate is required for the action of the respiratory enzymes as such, or that DNP does not completely circumvent transphosphorylation between adenine nucleotide and a hypothetical primary ester.

SUMMARY

1. The enzymes catalysing the esterification of inorganic phosphate coincident with the oxidation of glutamate have been found to be localized in the mitochondrial fraction of liver and kidney. Phosphate esterification coupled with fatty acid oxidation has been demonstrated in preparations of rat-liver mitochondria.

2. The depression of oxidative phosphorylation by dinitrophenol (DNP) has been confirmed using a number of substrates. Two metabolic products of DNP were found to be without influence on oxidative phosphorylation, which was markedly inhibited, however, by low concentrations of a number of dyestuffs and also by 1:11-diamidinoundecane.

3. The degree of stimulation by DNP of the oxidation of glutamate by kidney particles deficient in inorganic phosphate and/or adenine nucleotide has been found to be strongly dependent on the presence of fluoride in the suspension medium. In general, substances which depress oxidative phosphorylation also 'replace' inorganic phosphate and/or adenine nucleotide in this system.

4. Acetate oxidation catalysed by kidney particles is inhibited by substances which disrupt the linkage between oxidation and phosphorylation. The inhibition of acetate oxidation by DNP is considerably diminished in the presence of fluoride.

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REFERENCES

Bailey, K. (1942). *Biochem. J.* **36**, 121.
Bailey, K. & Webb, E. C. (1948). *Biochem. J.* **42**, 60.
Berger, L., Slein, M. W., Colowick, S. P. & Cori, C. F. (1946). *J. gen. Physiol.* **29**, 379.
Cohn, G. & Kolthoff, I. M. (1942). *Industr. Engng Chem.* (Anal. ed.), **14**, 885.
Cross, R. J., Covo, G. A., Taggart, J. V. & Green, D. E. (1949). *J. biol. Chem.* **177**, 655.
Dickens, F. (1936*a*). *Biochem. J.* **30**, 1064.
Dickens, F. (1936*b*). *Biochem. J.* **30**, 1233.
Dickens, F. (1939). *Biochem. J.* **33**, 2017.
Dodds, E. C. & Greville, G. D. (1934). *Lancet*, **1**, 398.
Dounce, A. L., Rothstein, A., Beyer, G. T., Meier, R. & Freer, R. M. (1948). *J. biol. Chem.* **174**, 361.
Dubois, K. P. & Potter, V. R. (1943). *J. biol. Chem.* **147**, 41.
Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
Friedheim, E. A. H. (1934). *Biochem. J.* **28**, 173.
Goranson, E. S. & Erulkar, S. P. (1949). *Arch. Biochem.* **24**, 40.
Green, D. E., Loomis, W. H. & Auerbach, V. H. (1948). *J. biol. Chem.* **172**, 389.
Guerbet, M. & Meyer, A. (1932). *Ann. Physiol. Physicochim. biol.* **8**, 117.
Harrop, G. A. & Barron, E. S. G. (1928). *J. exp. Med.* **48**, 207.
Hogeboom, G. H., Schneider, W. C. & Pallade, G. E. (1948). *J. biol. Chem.* **172**, 619.
Johnson, M. J. (1941). *Science*, **94**, 200.
Judah, J. D. (1949). *Brit. J. Pharmacol.* **4**, 120.
Keilin, D. & Hartree, E. F. (1937). *Proc. roy. Soc.* B, **122**, 298.
Kennedy, E. P. & Lehninger, A. L. (1948). *J. biol. Chem.* **172**, 847.
Kennedy, E. P. & Lehninger, A. L. (1949). *J. biol. Chem.* **179**, 957.
Kornberg, A. & Lindberg, O. (1948). *J. biol. Chem.* **176**, 665.
Kornberg, A. & Pricer, W. E. (1950). *J. biol. Chem.* **182**, 763.
Lardy, H. A. & Elvehjem, C. A. (1945). *Ann. Rev. Biochem.* **14**, 1.
Loomis, W. F. & Lipmann, F. (1948). *J. biol. Chem.* **173**, 807.
Lowry, O. H. & Lopez, J. A. (1946). *J. biol. Chem.* **162**, 421.

Lynen, F. (1941). *Liebigs Ann.* **546**, 120.
McElroy, W. D. (1947). *Quart. Rev. Biol.* **22**, 25.
McIlwain, H. (1949). *Biochem. J.* **44**, xxxiii.
McIlwain, H. & Rodnight, R. (1949). *Biochem. J.* **44**, 470.
Meyerhof, O. & Geliazkowa, N. (1947). *Arch. Biochem.* **12**, 405.
Michaelis, L. (1931). *Biochem. Z.* **234**, 139.
Nelson, N. (1944). *J. biol. Chem.* **153**, 375.
Ochoa, S. (1943). *J. biol. Chem.* **151**, 493.
Ogston, A. G. & Smithies, O. (1948). *Physiol. Rev.* **28**, 283.
Peiss, C. N. & Field, J. (1948). *J. biol. Chem.* **175**, 49.
Polis, D., Polis, E., Kerrigan, M. & Jedeikin, L. (1949). *Arch. Biochem.* **23**, 505.
Potter, V. R. (1945). *Arch. Biochem.* **6**, 439.
Potter, V. R. (1947). *J. biol. Chem.* **169**, 17.
Rossiter, R. J. (1940). *J. Endocrinol.* **2**, 165.
Schneider, W. C. (1948). *J. biol. Chem.* **176**, 259.
Seits, I. E. & Engelhardt, V. A. (1949). *C.R. Acad. Sci., U.R.S.S.* **66**, 439.
Slater, E. C. (1949). *Biochem. J.* **45**, 8.
van Heyningen, R. (1942). Report to the Ministry of Supply, no. 10, by M. Dixon.
Williamson, S. & Green, D. E. (1940). *J. biol. Chem.* **135**, 345.
Young, L. (1937). *J. biol. Chem.* **120**, 659.

The Use of Organic Solvents at Low Temperature for the Separation of Enzymes. Application to Aqueous Rabbit Muscle Extract

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Enzymes may be separated and purified by a number of methods, such as fractional adsorption and precipitation. The most widely used method has been that of salting out the proteins with neutral salts (cf. review of the literature by Cohn, Strong, Hughes, Mulford, Ashworth, Melin & Taylor, 1946). This method is safe, but the salting-out coefficient depends on rather unspecific characteristics, such as the shape and size of the protein molecule.

Organic solvents have not been applied so generally for the separation of enzymes, since they denature proteins at room temperature. During the war, Cohn, Leutscher, Oncley, Armstrong & Davis (1940) and Cohn *et al.* (1946) extensively developed the ethanol precipitation method of Mellanby (1908) and Hardy & Gardiner (1910). By working at low temperatures and low ionic strengths numerous protein fractions were isolated from blood plasma. Ethanol fractionation has various advantages over the salting-out method as pointed out by Edsall (1947). At low ionic strengths, the interaction between proteins and salts depends on specific properties of protein molecules, such as the distribution of electric charges. Small variations in dielectric constant and ionic strength will produce large and specific changes in the solubilities of proteins.

The present investigation was undertaken to study the separation of enzymes in tissue extracts from water-organic solvent mixtures. Enzyme tests provide a simpler and more specific measure of purity than the usual physical methods (such as solubility measurements and electrophoresis) which were applied by Cohn *et al.* (1946). Simple activity determinations will show where a protein is separating out and how much denaturation (as measured by loss of biological activity) results from the fractionation.

Aqueous extract of rabbit muscle was chosen as source of enzymes. No systematic protein fractionation of rabbit-muscle extract has been reported, although much is known about its enzyme systems and a number of studies have appeared recently, e.g. salting-out curves (Distèche, 1948), electrophoretic patterns (Jacob, 1947, 1948; Dubuisson, 1950), and ultracentrifugal studies (Amberson, Erdös, Chinn & Ludes, 1949). Most of the glycolysis enzymes are part of the myogen complex with albumin-like properties and a very high solubility in water-salt mixtures. For these enzymes separation by organic solvents, with a lowered dielectric constant, should be useful.

METHODS

Preparation of rabbit-muscle extract

Minced rabbit muscle was extracted at 0° with an equal volume of distilled water for 20 min. with gentle stirring. After centrifuging for 15 min., the supernatant was dialysed first against cold running water for 3 hr., and then against 20 times its volume of distilled water at 4° for 6 hr. The conductivity then equalled that of about 0·007 M-KCl. The pH was adjusted to the required value with 0·05 M-acetate buffer (pH 4), or $NaHCO_3$. This extract will be referred to as 'original extract' in the text.

Fractionation with organic solvents, at 0° and below

The insoluble precipitate formed on dialysis (ppt. *D*, containing some myosin, globulin *X* and denatured proteins) was removed by centrifugation. Fractionation of the supernatant was carried out in a metal vessel to permit rapid heat

exchange. The organic solvent was added through a capillary jet and permitted to flow down the cold wall of the container slowly while the solution was kept rapidly stirred avoiding froth (Fig. 1). As soon as enough organic solvent had been added to keep the extract from freezing the temperature of the bath was lowered from 0° to −3°. When the solution contained 20 or 40% (v/v) of organic solvent, the temperature was reduced to −5° and −7°, respectively. Since long standing in the presence of organic solvents tends to denature enzymes even at low temperatures, the precipitate was centrifuged down, after 20 min. equilibration, in a cold

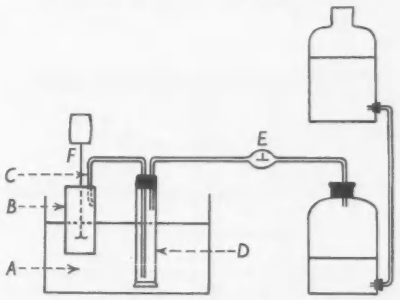

Fig. 1. Apparatus used for fractionations with organic solvents. *A*, cold bath; *B*, aluminium vessel; *C*, capillary tubing; *D*, graduated cylinder, containing the organic solvent; *E*, three-way tap; *F*, stirrer.

centrifuge set at the same temperature as the cold bath. The precipitates were taken up in a small volume of dilute buffer and dialysed overnight at 4° against 0·033 M-phosphate buffer (pH 7·5). The fractions were then 'freeze-dried' and weighed. They could be kept for indefinite periods. After several fractionations it was found advantageous to compensate for dilution as well as removal of ions by the protein precipitate by adding 3 M-sodium acetate (at pH 6·5) to give a final concentration of 0·015 M, when the organic solvent level reached 40% (v/v). This was done in the case of the *n*-propanol fractionation.

Enzyme estimations

For each test the enzyme fractions were diluted sufficiently for the rate of reaction to be proportional to the enzyme concentration. All phosphate determinations were carried out by the method of Fiske & Subbarow (1925) using a King photoelectric absorptiometer. As a measure of protein concentration, the absorption density at 278 mμ. was measured in the Beckman spectrophotometer; the purification achieved was calculated by comparing the activity (units of enzyme/ml./absorption density at 278 mμ.) of a particular fraction with that of the original extract.

Adenosinetriphosphatase. The activity of Ca-activated adenosinetriphosphatase was tested in glycine buffer (pH 9·0) as described by Bailey (1949).

Myokinase. Increases in inorganic phosphate were determined after incubating at 28° (*a*) the enzyme fraction + myosin + 0·0017 M-adenosinediphosphate (ADP) + 0·007 M-Ca^{++} in glycine buffer pH 9·0 (total volume 1·5 ml.); (*b*) as control the same mixture as (*a*) without the enzyme fraction. After 20 min. the reaction was stopped with 1 ml. 10% trichloroacetic acid and the inorganic phosphate determined.

Phosphorylase. The method of Cori, Cori & Green (1943) was followed using glucose-1-phosphate as substrate. It was necessary to add adenylic acid since most of the phosphorylase was present as the phosphorylase *b* of Cori *et al.* (1943).

Lactic dehydrogenase. The enzyme was tested in 0·05 M-KCN (neutralized), 0·067 M-sodium lactate, coenzyme I (1 mg./ml. of reaction mixture, about 50% pure) and 0·05 M-phosphate buffer (pH 7·4). The rate of reduction of coenzyme I was measured at 20° in the Beckman spectrophotometer by increase of absorption at 340 mμ.

Adenylic deaminase. The reaction mixture consisted of 0·05 M-phosphate buffer pH 6·0, 0·002 M-adenylic acid (neutralized to pH 6·0) and 0·5 mg. of enzyme/ml. Samples of 1 ml. were removed at zero time and after 5 and 10 min. of incubation at 38°. They were added to 0·2 ml. Nessler reagent to stop the reaction, then suitably diluted and the intensity of the colour complex formed with the liberated NH_3 measured in the Beckman spectrophotometer at 420 mμ. in glass cells.

Aldolase. The rate of breakdown of hexosediphosphate was determined according to the chemical method described by Taylor, Green & Cori (1948).

Enolase. The rate of formation of phospho-enolpyruvic acid from 2-phosphoglyceric acid was measured in the Beckman spectrophotometer by change of absorption at 240 mμ. (Warburg & Christian, 1941). 2-Phosphoglyceric acid was prepared by Kiessling's method from sodium β-phosphoglycerate, as described by Warburg & Christian (1941).

Creatinephosphokinase. The synthesis of phosphocreatine from adenosinetriphosphate (ATP) and creatine was determined as follows: the reaction mixture consisted of 0·05 M-glycine buffer at pH 8·8, 0·05 M-NaF (to inhibit at least partly any adenosinetriphosphatase and phosphatase present), 0·005 M-Mg^{++}, 0·02 M-creatine, 0·03 M-cysteine (neutralized), 0·005 M-ATP and the enzyme fraction. Samples (1 ml.) were removed at zero time and after 2 and 5 min. incubation at 30°, and added to 5 ml. trichloroacetic acid. Inorganic phosphate was determined after the hydrolysis of phosphocreatine in N-H_2SO_4 in the presence of molybdate for 45 min. at room temperature (Fiske & Subbarow, 1929). An increase in phosphate was due to the formation of phosphocreatine, since the values remained unchanged under the same conditions in the absence of creatine.

Electrophoresis. The original extract and 1·5% solutions of several fractions were dialysed overnight against 0·033 M-phosphate buffer pH 7·5 (*I*, 0·09), and the electrophoresis carried out in a Tiselius apparatus at 4°, with a potential gradient of 7·5 V./cm.

RESULTS

Factors affecting the protein precipitation

The main factors affecting the solubility of proteins have to be carefully controlled during the experimental procedure. They are temperature, protein concentration, ionic strength, pH and dielectric constant. The variation in temperature was the same in all experiments (0 to −7°) and the protein concentration of the extracts was fairly constant (2·5–3% protein). The effect of varying the other three factors was studied to establish the best overall conditions for sharp separation with the least amount of denaturation.

Variation of ionic strength. Fig. 2 (*a*) shows how the proteins precipitate out when ethanol is added at pH 6·5. Since muscle contains many protein components, and there is some overlapping in the different fractions, a smooth precipitation curve is obtained. In one case the ionic strength of the extract was very low (after 9 hr. of dialysis) and the conductivity of the solution equalled that of 0·007 M-potassium chloride. The exact ionic strength cannot be given since the proportion of the different ions was not known, but it could not be more than 0·03.

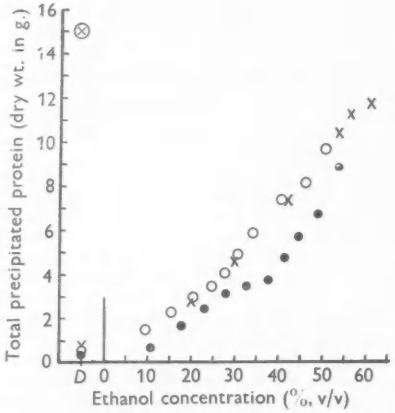

Fig. 2*a*. Protein precipitation curves, using ethanol at pH 6·5. ○, ×, two separate fractionations, $I<0{\cdot}03$; ●, $I=0{\cdot}1$ at beginning of experiment. Ordinates represent the weight of the precipitated protein fractions, freeze-dried after dialysis. Each fraction contains a small amount of phosphate (accounting for less than 5% of the total weight). ⊗ = amount of freeze-dried protein present in the original extract (500 ml.); *D*, precipitate removed after dialysis, before adding ethanol.

When the ionic strength was lowered by further dialysis, any precipitate formed by ethanol was too fine to be centrifuged down. In the lower curve the ionic strength had been raised to 0·1 with phosphate buffer at the beginning of the experiment. As may be expected the proteins are more soluble, and a greater ethanol concentration is required to precipitate a given amount of protein. Under these conditions more denaturation results, and also the enzymes are found to be less sharply separated than at the lower ionic strength.

Variation in pH. A pH around 6·5 appears to be most favourable for protein precipitation. At both pH 5 and 8 the proteins are more soluble so that at a concentration of 50 % (v/v) ethanol only half of the protein has come out of solution (Fig. 2*b*). Enzymic tests show that there are great losses by denaturation and poor separation of the different components at pH 5 and 8.

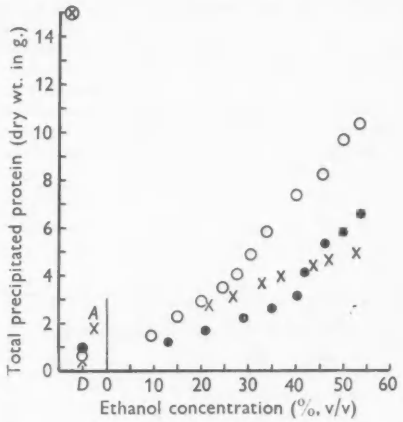

Fig. 2*b*. Protein precipitation curves using ethanol at an ionic strength around 0·03 or less. ●, pH 8·0; ○, pH 6·5; ×, pH 5·0. ⊗, *D*, as in Fig. 2*a*. *A*, precipitate formed on acidifying to pH 5·0. The weights of *A* and *D* are included in the ethanol precipitation curves.

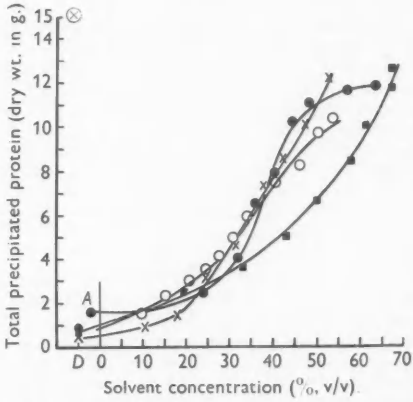

Fig. 3. Protein precipitation curves, using various organic solvents at pH 6·5 and $I<0{\cdot}03$. ■, methanol; ○, 97% ethanol; ×, *n*-propanol; ●, acetone (A.R.). ⊗, *D*, *A*, as in Figs. 2*a*, *b*.

Variation in dielectric constant. The dielectric constant was varied by using a number of different organic solvents (alcohols with one, two or three carbon atoms, and acetone) while the ionic strength and pH were kept constant (Fig. 3). Methanol has the highest dielectric constant of these and the proteins are most soluble in methanol-water mixtures. The other three curves are fairly similar; acetone

gives the steepest precipitation curve, but this flattens out before all proteins have come out of solution. Denaturation is not proportional to the chain length; *n*-propanol produces most denaturation, next comes methanol, and then ethanol. To avoid denaturation, it is best to keep the concentration of organic solvent as low as possible.

From these results it was evident that a pH of about 6·5 and a very low ionic strength (approx. 0·03) provided the most favourable conditions for fractionating the proteins, and these were adopted for the enzymic studies.

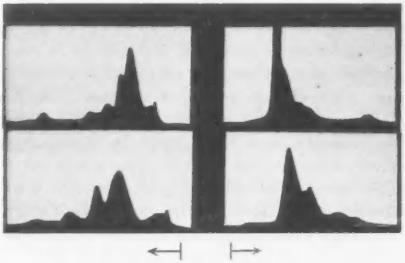

Fig. 4. Electrophoretic pattern (anodic) of aqueous extract of rabbit muscle dialysed against phosphate buffer pH 7·5, $I = 0{\cdot}09$. The arrows below the picture indicate the position of the boundary at zero time and the direction of movement. The pictures were taken after 1 and 2 hr. (7·5 V./cm.). Left, ascending boundary; right, descending boundary.

Fig. 4 shows the electrophoretic pattern of the original extract. At least eight components are visible, but muscle extract is known to contain many more different enzymes.

Separation and purification of enzymes

Seven enzymes were tested and Fig. 5 shows how they separated out with ethanol. The percentage of enzyme activity recovered in a particular fraction was plotted against the ethanol concentration (as percentage v/v) at which this fraction had precipitated out. The method appeared to be better for some enzymes than for others. Aldolase, for example, precipitated over a wide range of ethanol concentration with hardly any purification. On the other hand, 86 % of the phosphorylase was recovered between 10 and 20 % (v/v) ethanol with a 12-fold purification in one step. Adenosinetriphosphatase and adenylic deaminase were removed from the preparation at very low ethanol concentrations, i.e. 15 % (v/v). This is useful because adenosinetriphosphatase is usually difficult to get rid of and is apt to interfere with enzymic tests using the adenylic system. Enolase and creatinephosphokinase were

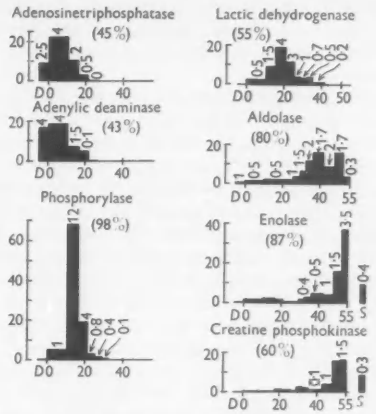

Fig. 5. Separation of enzymes by an ethanol fractionation, pH 6·5, $I < 0{\cdot}03$. Ordinates, percentage of the original amount of enzyme activity appearing in each fraction. Abscissae, ethanol concentration (%, v/v). Numbers above histograms indicate the purification achieved compared with the original extract (enzyme activity being taken as units/ml./adsorption density at 278 mμ.). Figures in brackets represent the total percentage recovery of the enzyme in all the fractions. *D*, precipitate formed on dialysis before ethanol addition. *S*, supernatant after centrifuging down the last precipitate.

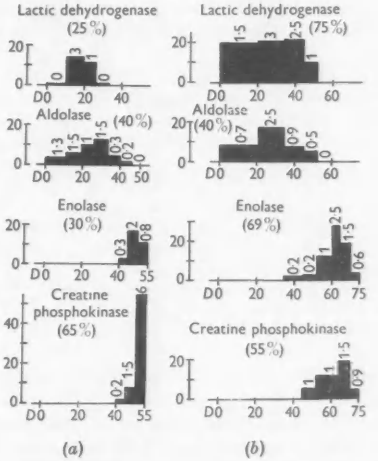

Fig. 6. (*a*) Separation of enzymes by a fractionation with *n*-propanol, pH 6·5, $I < 0{\cdot}03$. (*b*) Separation of enzymes by a methanol fractionation, pH 6·5, $I < 0{\cdot}03$. Ordinates, percentage of the original amount of enzyme activity appearing in each fraction. Abscissae, alcohol concentration (%, v/v). Numbers above histograms, figures in brackets and *D* as in Fig. 5.

the most soluble of the enzymes tested in ethanol-water mixtures. They came out of solution with the last protein fraction which could be precipitated.

Methanol (Fig. 6*b*) produces hardly any separation or purification, and is the least useful of the solvents tried. Fig. 6*a* gives the results from a fractionation with *n*-propanol. This solvent leads to a good separation in some cases, but the recovery of enzyme activity is too low for general use. However, propanol may be very useful for some enzymes which are particularly stable to alcohol, such as creatine-phosphokinase. Of this enzyme 55 % was recovered in one *n*-propanol fraction with a sixfold purification. This sample was free of adenosinetriphosphatase, adenylic deaminase and myokinase.

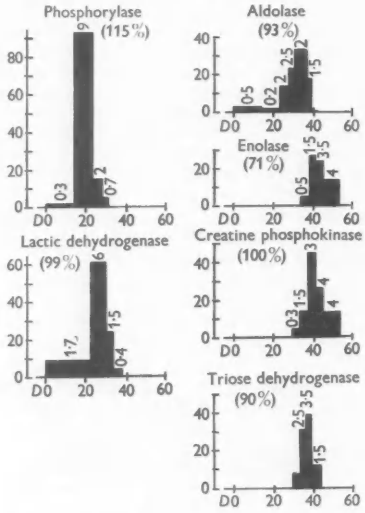

Fig. 7. Separation of enzymes by an acetone fractionation, pH 6·5, $I < 0{\cdot}03$. Ordinates, percentage of the original amount of enzyme activity appearing in each fraction. Abscissae, acetone concentration (%, v/v). Numbers above histograms, figures in brackets and *D* as in Fig. 5.

Acetone (Fig. 7) seems to be superior to the other solvents for enzyme separation. Since the precipitation curve is steep it is essential to take very small steps (at times a 2 vol. % increase is sufficient). An almost complete recovery and fairly sharp separation is obtained with most of the enzymes tested, and with the precautions mentioned the products are completely soluble in water. With both acetone and ethanol the results are easily reproducible as long as the temperature, ionic strength, pH and dielectric constant are carefully controlled.

DISCUSSION

The above results, obtained from one stage of fractionation of crude muscle extract, have shown that the use of pure acetone with the strict temperature precautions described, at about pH 6·5 and an ionic strength of approximately 0·03, provides the most favourable conditions for enzyme separations. The advantages of the use of organic solvents appear to be that generally the separation is sharper than with ammonium sulphate, where the enzymes tend to spread over wide salt concentrations. Since any lipid material goes into solution, the products from an acetone fractionation tend to be much cleaner than those from an ammonium sulphate fractionation and they are soluble in water. A number of enzymes may be prepared from the same extract with almost 100 % recoveries. The above conditions may probably also be applied to other tissue extracts and they may be recommended especially for the first step in fractionations, with possible changes in pH and ionic strength for sub-fractionations, or followed by ammonium sulphate precipitation and adsorption on calcium phosphate gel.

It is not possible to say why some enzymes show more tendency to spread over a number of fractions than others. The spreading may be due to protein interactions and the adsorption of proteins on one another. Too little is known about these factors to control them at this stage. Adenylic deaminase, for example, precipitates with adenosinetriphosphatase, with the amount of purification running roughly parallel in the different fractions, although it is not certain whether the two are identical proteins. Hermann & Josepovits (1949) have not been able to separate them, but according to Ferdman & Nachiropenko (1946) some of the adenylic deaminase stays in the supernatant if myosin is reprecipitated.

Both the nature and concentration of electrolytes influence the fractionation profoundly. The presence of some electrolytes is necessary to protect the proteins from denaturation and to help form complexes favouring precipitation. On the other hand, an increase in ionic strength from 0·03 to 0·1 decreases the sharpness of separation and the recovery of enzyme activity considerably. The nature of the ions added has to be carefully considered. Phosphate tends to keep the proteins in solution longer; this may be due to a specific interaction of phosphate with the muscle proteins. A trace of calcium is at times useful to decrease the solubility of some proteins, or to flocculate very fine precipitates. Negative ions may be of importance in forming complexes with the proteins; a small amount of acetate, for example, has in some cases increased the recovery of an enzyme. Not enough work has been done to

determine whether this effect is general or is due to a specific protection.

Several conductivity measurements (Table 1) have shown that the conductivity of the muscle extract after precipitating some proteins with ethanol is lower than that of a pure calcium chloride solution to which an equivalent amount of ethanol had been added. Thus dilution with organic solvents as well as the formation of protein precipitates will deplete the solution of salts. The addition of sodium acetate at pH 6·5 to give a final concentration of 0·015 M when the organic solvent level reached 40 % (v/v) was found useful in the *n*-propanol fractionation and it is recommended that some salt should be added with the organic solvent to avoid depletion.

Table 1. *Effect of protein precipitation by means of ethanol on the conductivity of solutions*

	Ethanol concn. (%, v/v)	Specific conductance (expressed as equivalent molarity of KCl)
Aqueous muscle extract (dialysed 20 hr.)	0	0·002
Dialysed muscle extract containing 0·0036 M-$CaCl_2$	0	0·0085
Dialysed muscle extract containing 0·0036 M-$CaCl_2$	20	0·0038
Dialysed muscle extract containing 0·0036 M-$CaCl_2$	40	0·0019
0·0045 M-$CaCl_2$ solution	0	0·00805
0·0045 M-$CaCl_2$ solution	20	0·0051
0·0045 M-$CaCl_2$ solution	40	0·0033

It was found that the enzymes precipitated out of solution in a definite order, i.e. each enzyme tended to come out of solution when a constant percentage of the total protein had precipitated, regardless of the alcohol concentration at which this level of precipitation may be reached. This order was constant under the conditions of pH and ionic strength tested in the present study or after the addition of lysine (3 g./100 ml.).

A number of the fractions obtained were analysed electrophoretically at pH 7·5 in phosphate buffer (*I*, 0·09). Except for myoalbumin, the most rapid component, it was found that the faster moving components were precipitated by lower alcohol concentrations than the slower components. There seemed to be a rough parallelism between mobilities and the concentrations of alcohol needed for precipitation. These findings are opposite to the results on blood plasma (Cohn *et al.* 1946), where the proteins with the lowest mobilities were the most insoluble in alcohol-water mixtures and were precipitated out first.

Rabbit-muscle extract consists of such a complex mixture of proteins that it was not possible to identify all the peaks on the electrophoretic picture (Fig. 4). The individual fractions were not sufficiently homogeneous, and only seven of the numerous enzymes were tested for. The problem is complicated by the fact that a protein will not necessarily move at the same rate when pure, as in the presence of a large number of other components. The difficulties in trying to establish the relationship between the classical muscle proteins and the peaks have been discussed by Jacob (1947). Aldolase, phosphorylase and triosephosphate dehydrogenase have been placed by mobility determinations on crystalline enzymes (Taylor *et al.* 1948; Green, 1945; Cori, Stein & Cori, 1948). From the present study it may be concluded that adenylic deaminase, adenosinetriphosphatase, phosphorylase and lactic dehydrogenase are among the smaller and faster moving components. Most of the other glycolysis enzymes (enolase, aldolase, triosephosphate dehydrogenase, creatinephosphokinase) form part of the large complex of proteins moving most slowly at pH 7·5 (components *l* and *m* of Jacob, 1947).

SUMMARY

1. A fractionation of the water-soluble proteins of rabbit muscle has been carried out using organic solvents at low temperatures and low ionic strengths. Enzyme estimations were used to follow the separation of the proteins.

2. The following conditions were, in general, found best for sharp separation of the enzymes with the least amount of denaturation: precipitation of the proteins with acetone at pH 6·5, at very low ionic strengths (around 0·03) and low temperatures (0° and below).

3. The results obtained are easily reproducible and a four- to five-fold purification in one stage is achieved on the average. The procedure compares favourably with other methods of enzyme purification.

4. Most of the glycolysis enzymes separated out free from adenylic deaminase and adenosinetriphosphatase. Creatinephosphokinase was obtained free from myokinase.

This problem was suggested by Dr M. Dixon, F.R.S., to whom I am very grateful for his advice and interest. I should also like to thank Mr K. McQuillen for carrying out the conductivity measurements; Mr J. Morgan for preparing the crude muscle extracts and for samples of adenosinetriphosphate; and the Medical Research Council for a grant.

REFERENCES

Amberson, W. R., Erdös, T., Chinn, B. & Ludes, H. (1949). *J. biol. Chem.* **181**, 405.
Bailey, K. (1949). *Biochem. J.* **45**, 479.
Cohn, E. J., Leutscher, J. A., Oncley, J. L., Armstrong, S. H. & Davis, B. D. (1940). *J. Amer. chem. Soc.* **62**, 3396.
Cohn, E. J., Strong, L. E., Hughes, W. L., Mulford, D. J., Ashworth, J. N., Melin, M. & Taylor, H. L. (1946). *J. Amer. chem. Soc.* **68**, 459.
Cori, C. F., Cori, G. T. & Green, A. A. (1943). *J. biol. Chem.* **151**, 31, 39.
Cori, G. T., Stein, M. W. & Cori, C. F. (1948). *J. biol. Chem.* **173**, 605.
Distèche, A. (1948). *Biochim. Biophys. Acta*, **2**, 265.
Dubuisson, M. (1950). *Biol. Rev.* **25**, 46.
Edsall, J. T. (1947). *Advanc. prot. Chem.* **3**, 384.
Ferdman, D. & Nachiropenko, S. (1946). *Ukr. biochem. J.* **18**, 110.
Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
Fiske, C. H. & Subbarow, Y. (1929). *J. biol. Chem.* **81**, 629.
Green, A. A. (1945). *J. biol. Chem.* **158**, 315.
Hardy, W. B. & Gardiner, S. (1910). *J. Physiol.* **40**, lxviii.
Hermann, V. S. & Josepovits, G. (1949). *Nature, Lond.*, **164**, 845.
Jacob, J. (1947). *Biochem. J.* **41**, 83.
Jacob, J. (1948). *Biochem. J.* **42**, 71.
Mellanby, J. (1908). *Proc. roy. Soc.* B, **80**, 399.
Taylor, J. F., Green, A. A. & Cori, G. T. (1948). *J. biol. Chem.* **173**, 591.
Warburg, O. & Christian, W. (1941). *Biochem. Z.* **310**, 384.

The Preparation of Permanent Turbidimetric Standards for Use in the Determination of Small Amounts of Chloride

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Permanent turbidimetric standards are useful in the determination of small amounts of chloride. They are of particular value in the control of the purity of Analar and B.P. chemicals, since they avoid the repeated and costly preparation of fresh standards; and they may be used for other turbidimetric tests, e.g. the determination of proteins by precipitation with sulphosalicylic or trichloroacetic acid.

We feel that such standards may be of value to workers in biochemical and other fields, and the purpose of this paper is to describe the preparation of permanent standards of cloudy Perspex which we have found useful in the routine determination of chloride, and which have been employed by King (1950) for urinary and C.S.F. protein estimations.

The method of preparation of the standards involves, in the first place, the production of a clear interpolymer of 50% methyl methacrylate and 50% styrene. In the second stage of the process, this interpolymer is dissolved in methyl methacrylate monomer; this monomer is then polymerized under conditions which involve uniform precipitation of the styrene/methyl methacrylate interpolymer.

METHODS

Interpolymer. A polymethyl methacrylate-polystyrene interpolymer is prepared as follows: 200 g. of redistilled methyl methacrylate monomer are heated with 200 g. of redistilled styrene monomer and 1·6 g. benzoyl peroxide catalyst with stirring in a conical flask (600 ml.) at 70° under reflux, until the viscosity of the syrup is approximately 4 poises. This operation takes about three-quarters of an hour. The syrup is cooled and then evacuated free from air by means of a water pump. The cold syrup is poured into a cell formed by two glass plates (30·5 × 30·5 × 0·6 cm.) separated from one another by rubber tubing (6 mm. diam., 1 mm. wall) and bound by screw clips (Fig. 1). After filling and binding, the cell is maintained at 75° for about 6 hr. in an air oven. A steady current of air is circulated through the oven throughout this period. After cooling, the polymer sheet is detached from the glass plates, ground and bottled.

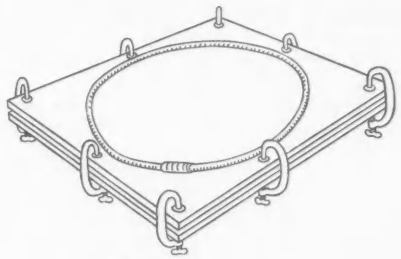

Fig. 1. Cell for forming interpolymer.

Standards. Turbidimetric standards are prepared in the following way: a known weight of the ground polymer (see below) is dissolved in 50 ml. of redistilled methyl methacrylate monomer by heating under reflux with stirring for 1 hr. at 50° in the apparatus shown in Fig. 2. 1·1 ml. of a

solution of 0·5 g. of benzoyl peroxide in 10 ml. of methyl methacrylate monomer is then added. The temperature is raised to 70–85° and maintained there until a syrup of viscosity 4 poises is produced: this operation takes approximately three-quarters of an hour. The syrup is cooled, evacuated free

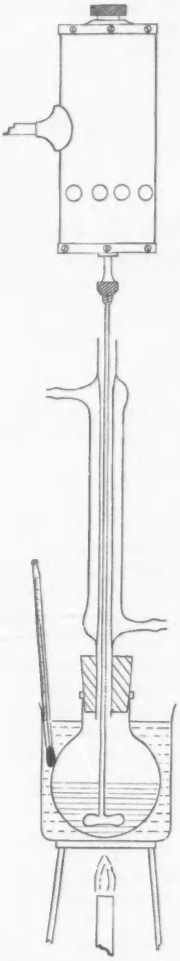

Fig. 2. Apparatus for preparation of interpolymer-monomer syrup.

from air and poured into soda-glass test tubes (25 × 1 cm.). These tubes are then lowered at a rate of 25 cm. in 12 hr. into an oil bath maintained at 75°, using the apparatus shown in Fig. 3. The purpose of carrying out the final polymerization by this method is to avoid, as far as possible, contraction bubbles in the final product.

When fully immersed the tubes are allowed to remain in the oil bath for a further period of 4 hr., in order to obtain the desired turbidimetric standards. The glass tubes are broken, and the prepared standards cut into suitable lengths (5–7·5 cm.) and fixed in tightly fitting glass tubes (1 cm. diam.).

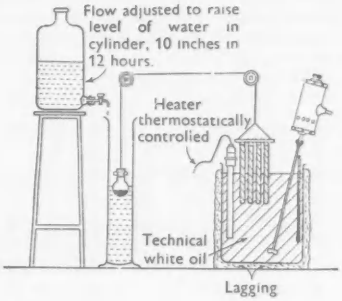
Flow adjusted to raise level of water in cylinder, 10 inches in 12 hours.
Heater thermostatically controlled
Technical white oil
Lagging

Fig. 3. Apparatus for preparation of standards.

The turbidity produced varies with the concentration of the interpolymer in the product, but in our experience not in a definite and reproducible manner. By dissolving amounts of polymethyl methacrylate-polystyrene interpolymers, ranging from 0·15 to 0·42 g., in 50 ml. methyl methacrylate monomer we have prepared a range of ten standards very suitable for chloride tests. These standards cover the range 0–3·8 ml. 0·01 N-HCl when used according to the method given below, i.e. using 1 g. of a substance for analysis the standards would cover the range 0–0·135% chlorine as chloride.

Comparison. We are indebted to Prof. E. J. King for his advice on the method of matching AgCl suspensions with turbidimetric standards. When the standards are placed in a specially constructed viewing rack (Fig. 4) and illuminated by light from a window behind the operator, the turbidimetric comparisons are made very readily.

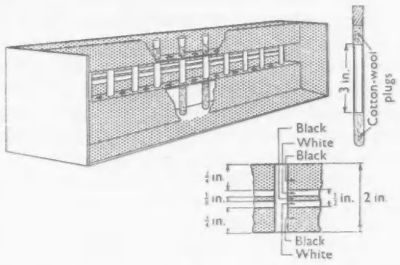

Fig. 4. Viewing rack for turbidimetric standards.

Calibration. It is, of course, necessary to calibrate against known AgCl suspensions. We have calibrated our standards by comparison with AgCl prepared as follows (B.P., 1948): a definite volume of 0·01 N-HCl and 1 ml. of HNO_3 (70%,

w/w) are measured into a 50 ml. flask. The solution is diluted to 50 ml. with distilled water, and 1 ml. of 5% (w/v) $AgNO_3$ is added. The turbid solution is set aside for 5 min., and then transferred, for viewing purposes, to tubes similar to those containing the permanent standards.

In our experience a standard prepared from 0·42 g. polymethyl methacrylate-polystyrene interpolymer and 50 ml. methyl methacrylate monomer corresponds to an AgCl suspension prepared in the above manner from 3·8 ml. 0·01 N-HCl.

SUMMARY

1. The preparation of turbidimetric standards, made of Perspex, has been described.

2. The turbidities of these standards are permanent, and form a useful basis of comparison for silver chloride, protein and other analytical procedures which depend on the production of finely dispersed precipitates.

REFERENCES

British Pharmacopoeia (1948), p. 757, Appendix 8A.

King, E. J. (1950). *Biochem. J.* **48**, 50.

Estimation of Protein in Urine and C.S.F. with Permanent Turbidimetric Standards of Perspex

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Several methods exist for the determination of the protein of urine, C.S.F. and other body fluids, including those of Esbach (1874), Exton (1925), Kingsbury, Clark, Williams & Post (1926) and Hiller, McIntosh & Van Slyke (1927). That of Kingsbury *et al.* is probably the simplest and the most widely used. Although it cannot be considered a precise method, it is nevertheless sufficiently accurate for clinical purposes. The results usually required for urinary and C.S.F. protein need be only approximate, and the assessment of the turbidity produced by mixing urine or C.S.F. with sulphosalicylic acid against convenient turbidimetric reference standards such as those of Kingsbury *et al.* makes a quick and very useful procedure for clinical laboratories. These standards, which consist of a permanent suspension of formazin in gelatin, were investigated by King & Haslewood (1936), and modified to allow the use of a small volume of the urine or C.S.F. The formazin standards are widely used; they are satisfactory within the limits imposed by the consistency of turbidity formation when protein and sulphosalicylic acid are mixed, and the ability of the observer to select the turbid standard which most closely matches the test mixture of urine or C.S.F. with the sulphosalicylic acid. Plimmer & Lowy (1945) found that even with a photoelectric photometer the agreement between the results of this procedure and those obtained by Kjeldahl was only about ±5%. With the visual matching of test with standards only about ±10% accuracy is obtained, but this is sufficient for the purpose.

Although the formazin standards have been easy to use, and have yielded acceptable results, they have not been as permanent as could have been wished. Despite careful stoppering and sealing of the test tubes in which they are contained, evaporation takes place, and the standards degenerate. With the object of securing more permanent standards the author approached Mr J. Haslam, who had already prepared for chloride estimation turbidimetric standards of solid rods of cloudy Perspex. It is believed that these standards are truly permanent in the sense that there has been no change in their optical characteristics over many months; and it is believed that they will remain unaltered indefinitely. The comparison of the protein-sulphosalicylic acid turbidities against these standards is as easy as it is against the formazin standards, and it is believed that they constitute a more satisfactory point of reference for this widely used procedure.

EXPERIMENTAL

The standards used were those described in the accompanying paper by Haslam & Squirrell (1950). The procedure is as follows:

Procedure. To 3 ml. of 3% (w/v) sulphosalicylic acid in a test tube of the same internal diameter as those which contain the standards is added 1 ml. of urine, C.S.F. or other albuminous body fluid (e.g. ascitic fluid). The turbidity is compared after 5 min. with those of the standards. If the degree of turbidity is too great for comparison a suitable dilution of the fluid is made with water, and the test is repeated. It is convenient to make the comparison by viewing the test and standards against a strip of white cardboard with a transverse black line (0·5 cm.) which is mounted in the rack as illustrated in Fig. 4 of Haslam & Squirrell (1950). The viewing is best made with the operator

Table 1. *Protein equivalents of a set of permanent turbidimetric standards*

(3 ml. of 3% sulphosalicylic acid plus 1 ml. protein solution.)

Polymer in standard (g.)	Protein equivalent of standard (mg./100 ml.)
0·150	6
0·225	10
0·235	15
0·275	20
0·300	27
0·310	35
0·325	42
0·375	50
0·400	60
0·42	75
0·45	85
0·48	100

Note. Each standard must be calibrated in terms of the sulphosalicylic acid-protein procedure, irrespective of the amount of polymer used. With the amount of polymer used here the turbidities are not in strict arithmetical ratio; but they fall approximately on a smooth curve.

By preparing a large number of standards it is possible to select those which show turbidities corresponding to protein concentrations of convenient selected amounts, e.g. 10, 20, ..., 90, 100 mg./100 ml.

standing with his back to a window and holding the rack of standards at eye level to be illuminated by daylight.

Calibration of the standards. This has been accomplished by preparing several dilutions of blood plasma whose protein content was accurately established by Kjeldahl determinations and treating them by the above procedure. Eight samples of normal blood plasma were used, and the average results for the Haslam & Squirrel standards are given in Table 1. The accuracy of the matched comparisons as judged by the Kjeldahl figures is about $\pm 10\%$, i.e. the average observer would assign a value to a 50 mg./100 ml. protein solution of anything between 45 and 55 mg./100 ml.

SUMMARY

The sulphosalicylic acid procedure for the turbidimetric estimation of protein has been successfully used with the permanent Perspex standards of Haslam & Squirrell (1950). The method yields only approximate results (about $\pm 10\%$); but is very useful for such purposes as the estimation of urine and C.S.F. protein in hospital laboratories. The standards are convenient to use, and appear to be permanent.

I am indebted to Mr J. Haslam for his ready co-operation and help in preparing the standards. My thanks are due to Miss V. Pash and Mr T. A. Webster for their valuable assistance in the calibration.

REFERENCES

Esbach, G. H. (1874). *C.R. Soc. Biol., Paris*, **1**, 33.
Exton, W. G. (1925). *J. Lab. Clin. Med.* **10**, 9.
Haslam, J. & Squirrell, D. C. M. (1950). *Biochem. J.* **48**, 48.
Hiller, A., McIntosh, J. F. & Van Slyke, D. D. (1927). *J. clin. Invest.* **4**, 235.
King, E. J. & Haslewood, G. A. D. (1936). *Lancet*, 2, 1153.
Kingsbury, F. B., Clark, C. P., Williams, G. & Post, A. L. (1926). *J. Lab. Clin. Med.* **11**, 981.
Plimmer, R. H. A. & Lowy, L. (1945). *Biochem. J.* **39**, xviii.

A Simplified Silver Iodate Method for the Determination of Chloride

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(*Received* 29 *June* 1950)

A method for the determination of chlorides in the blood and other biological fluids has been described by Haslewood & King (1936) and by Sendroy (1937; preliminary communication 1935). These methods are based on the reaction

$$NaCl + AgIO_3 \rightarrow AgCl + NaIO_3.$$

The sodium iodate formed is made to react with potassium iodide, added subsequently, from which three molecules of titratable iodine are liberated for each atom of chloride originally present; the final titration is with thiosulphate with starch as indicator.

The Haslewood & King procedure involved the preparation of a protein-free filtrate by Somogyi's (1930) zinc hydroxide method, the addition of an ammoniacal solution of silver iodate to this filtrate, subsequent removal of silver chloride and excess silver iodate by filtration of the solution after acidification with sulphuric acid, and titration of a sample of this filtrate with thiosulphate solution after the addition of potassium iodide.

Sendroy's procedure involved the precipitation of the plasma proteins with a special tungstic acid solution, filtration, and the shaking of the filtrate with a specially prepared purified solid silver iodate. The reaction between the dissolved sodium chloride and the silver iodate is complete in a very few minutes, and the silver chloride and excess silver

iodate are then filtered off. A sample of the filtrate is treated with potassium iodide and titrated with thiosulphate.

Van Slyke & Hiller (1947) simplified the Sendroy method by adding the solid silver iodate to the mixture of diluted plasma with sodium tungstate and phosphoric acid before removing the proteins, and then, after shaking, filtered off the proteins and the silver chloride and extra silver iodate in one operation. This method is very convenient and accurate, but it requires the preparation of the special silver iodate reagent of Sendroy (1939).

A modification of the Haslewood & King procedure has been developed with a view to reducing to a minimum the number of technical operations involved, and to using ordinary commercial silver iodate, which is used as before in ammoniacal solution, instead of the specially prepared solid silver iodate reagent of Sendroy. The proteins, silver chloride and excess silver iodate are precipitated together and removed by a single filtration, as in the Van Slyke & Hiller procedure. The method is quick and accurate; and the results are in good agreement with those obtained in parallel estimations carried out by the Van Slyke & Hiller method and by the mercuric nitrate method of Schales & Schales (1941).

EXPERIMENTAL

Solutions

Ammoniacal silver iodate solution. The stock solution consists of 1·8% (w/v) silver iodate ($AgIO_3$) dissolved in N-NH_3 solution. The working solution is prepared fresh before use by adding an equal volume of N-H_2SO_4 to, for example, 5 ml. of the stock in a centrifuge tube, and mixing well, to precipitate the $AgIO_3$. After centrifuging and pouring away the supernatant, the precipitate is dissolved in 0·3N-NH_3 equal in volume to that of the stock $AgIO_3$ solution taken. This precipitation and resolution ensure that the $AgIO_3$ used is free of soluble iodate.

N-*Ammonia solution.* 57 ml. conc. NH_3 (sp.gr. 0·882) to 1 l. with water.

0·3N-*Ammonia solution.* 17 ml. conc. NH_3 (sp.gr. 0·882) to 1 l. with water.

Tungstate-phosphoric acid reagent. 4·2 g. sodium tungstate ($Na_2WO_4.2H_2O$) are dissolved in 1 l. of 0·15M-H_3PO_4. The latter is prepared by making 10 ml. of conc. H_3PO_4 (sp.gr. 1·72) to 1 l. with distilled water.

Potassium iodide 2% (*w/v*).

0·005N-*Sodium thiosulphate.*

Method

To 0·2 ml. of plasma is added 0·5 ml. of ammoniacal $AgIO_3$ solution, followed by 3·3 ml. of tungstate-phosphoric acid reagent. The mixture is shaken well and filtered. (It is convenient to use a 7 cm. Whatman no. 42 filter paper.) The filtrate (1·0 ml.) is treated with 1·0 ml. of 2% KI solution and then titrated with 0·005N-$Na_2S_2O_3$ solution, using starch as indicator.

For determining the chloride in simple aqueous standard solutions the above procedure was found to be unsatisfactory, because of the presence of tungstate. For such solutions the method was modified by using 0·15M-H_3PO_4 instead of the tungstate-phosphoric acid reagent, thus: to 0·2 ml. of standard solution, 0·5 ml. of ammoniacal $AgIO_3$ solution was added and then 3·3 ml. of 0·15M-H_3PO_4, etc.

For whole blood the amount of sodium tungstate in the tungstate-phosphoric acid reagent should be doubled.

Table 1. *Comparison of chloride methods with standard solutions of sodium chloride*

Chloride present (mg. NaCl/100 ml.)	Chloride determined (mg. NaCl/100 ml.)		
	Present iodate method	Van Slyke & Hiller method	Schales & Schales method
305	305	305	301
	304	306	306
	305	307	306
450	448	454	449
	449	454	454
	450	455	454
607	607	603	—
	607	607	607
	—	607	—
750	746	745	744
	747	748	749
	748	748	755

Table 2. *Comparison of chloride methods with specimens of blood plasma*

Chloride determined (mg. NaCl/100 ml. blood)		
Present iodate method	Van Slyke & Hiller method	Schales & Schales method
618	617	618
618	610	618
618	616	612
595	593	585
598	594	591
600	595	591
602	601	596
605	600	607
605	604	612
607	604	612
608	607	—
626	625	629
628	626	633
628	627	633
631	628	—
371	367	369
372	368	379
373	368	379
377	371	389

RESULTS

The results obtained by this procedure for chloride analysis were compared with those obtained by two other methods of chloride determination, namely the iodometric method of Van Slyke & Hiller (1947) and

the mercurimetric method of Schales & Schales (1941). Tests were performed on both standard solutions and specimens of plasma. The results obtained are set out in Tables 1 and 2.

Results in the Schales & Schales method were calculated by comparison with a standard solution, the chloride content of which was 607 mg. sodium chloride/100 ml.

SUMMARY

A rapid and accurate method of estimating plasma chloride has been developed, using as a basis the silver iodate procedure of Haslewood & King (1936). The results obtained from this and the methods of Van Slyke & Hiller (1947) and Schales & Schales (1941) were in good agreement.

REFERENCES

Haslewood, G. A. D. & King, E. J. (1936). *Biochem. J.* **30**, 902.

Schales, O. & Schales, S. S. (1941). *J. biol. Chem.* **140**, 879.

Sendroy, J. Jun. (1937). *J. biol. Chem.* **120**, 335, 405, 419.

Sendroy, J. Jun. (1939). *J. biol. Chem.* **127**, 483.

Somogyi, M. (1930). *J. biol. Chem.* **86**, 655.

Van Slyke, D. D. & Hiller, A. (1947). *J. biol. Chem.* **167**, 107.

Studies in the Biochemistry of Micro-organisms

82. USTIC ACID, A METABOLIC PRODUCT OF *ASPERGILLUS USTUS* (BAINIER) THOM & CHURCH

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(*Received* 20 *June* 1950)

The *Aspergillus ustus* group of Aspergilli, as defined by Thom & Raper (1945) in their *Manual*, includes only two species, *A. ustus* (Bainier) Thom & Church and *A. granulosus* Raper & Thom. *A. ustus* is regarded by Thom & Raper as a single species aggregate including a whole series of interrelated individual strains differing markedly in their general habit, colony coloration and other characteristics. Representatives of the *A. ustus* group are perhaps the most abundant of all Aspergilli in the soil.

In spite of the ubiquity of *A. ustus* little was known until recently of its biochemical activities. Kurung (1945) reported that culture filtrates from a strain of *A. ustus* grown on a modified Czapek-Dox medium containing potassium chloride (0·5 g./l.) had marked antibacterial activity. Hogeboom & Craig (1946), in a continuation of this work, isolated two crystalline metabolic products containing chlorine, $C_{21}H_{17}O_6Cl_3$ (m.p. 185–187°) and $C_{21}H_{18}O_6Cl_2$ (m.p. 214–216°). Doering, Dubos, Noyce & Dreyfus (1946), in a parallel investigation, described other chlorine-containing metabolites of the same strain of *A. ustus* and gave the name ustin to the predominant component. Ustin was shown to be identical with Hogeboom & Craig's substance melting at 185–187°, but was assigned the formula $C_{19}H_{15}O_5Cl_3$.

We have found that four different strains of *A. ustus*, when grown on Raulin-Thom medium, which does not contain any chlorides, yield culture filtrates which give very intense colours with ferric chloride varying in shade from almost pure purple to purple-brown. In contrast, no comparable colours are given by culture filtrates of three of the same four strains of *A. ustus* when grown on Czapek-Dox medium. The metabolic product responsible for the purple colour has been isolated in a pure state and we propose the name *ustic acid* for it, since it has not been described previously. Pure ustic acid was isolated in yields of about 0·5 g./l. of culture filtrate from the best strain, grown on Raulin-Thom medium: the other three strains gave smaller yields. Ustic acid, $C_{11}H_{12}O_7$, forms colourless, rhombic prisms, m.p. 169–170° (decomp.). It has no optical activity. It contains one methoxyl group and is a strong acid giving a colourless crystalline potassium salt, $C_{11}H_{11}O_7K$. An aqueous solution of ustic acid gives an intense, stable, purple colour with aqueous ferric chloride; immediately decolorizes bromine water without the formation of a precipitate; does not give a precipitate with Brady's reagent (2:4-dinitrophenylhydrazine in 2N-hydrochloric acid) except after standing for some hours; and, at room temperature, rapidly reduces Fehling's solution, copper acetate solution and neutral copper sulphate solution with the formation of cuprous oxide in each case. On boiling with 2N-sulphuric acid, ustic acid is not decarboxylated, but is converted, with the loss of one molecule of water, into a colourless crystalline com-

pound, $C_{11}H_{10}O_6$, which may be conveniently described as *anhydro-ustic acid*, although it is, in fact, no longer acidic. This substance has very different properties from ustic acid. It gives a much less intense purple ferric chloride colour, does not reduce cold Fehling's solution and gives an immediate precipitate with Brady's reagent. Ustic acid cannot be regenerated from it by treatment of it with cold 2N-sodium hydroxide, which brings about deep-seated changes. It is of importance, however, since its formation explains the almost complete failure to obtain straight functional derivatives of ustic acid itself. Thus, methylation of ustic acid with diazomethane or with dimethyl sulphate and aqueous sodium hydroxide led to gums which could not be crystallized; with dimethyl sulphate and potassium carbonate in methanol a monomethyl ether, probably *ustic acid 4-methyl ether*, $C_{11}H_{11}O_6(OCH_3)$, was obtained; with dimethyl sulphate and potassium carbonate in acetone the *trimethyl ether* of *anhydro-ustic acid*, $C_{11}H_7O_3(OCH_3)_3$, insoluble in cold sodium hydroxide, resulted, identical in all its properties with the trimethyl ether obtained by direct methylation, with the same reagents, of anhydro-ustic acid. Similarly, while acetylation of ustic acid with acetic anhydride and anhydrous sodium acetate led only to an uncrystallizable gum, acetylation of either ustic acid or anhydro-ustic acid with acetic anhydride and pyridine yielded crystalline *triacetyl anhydro-ustic acid*, $C_{11}H_7O_3(O.CO.CH_3)_3$, which, unlike ustic acid itself, is insoluble in aqueous sodium bicarbonate. Finally, on treatment with Brady's reagent, ustic acid slowly gave and anhydro-ustic acid quickly gave the same *mono-2:4-dinitrophenylhydrazone*,

$$C_{11}H_{10}O_5(N.NH.C_6H_3(NO_2)_2).$$

In all these derivatives except ustic acid 4-methyl ether a molecule of water is lost from ustic acid in their formation.

As mentioned previously, ustic acid rapidly reduces copper sulphate in neutral aqueous solution at room temperature with the formation of cuprous oxide. The oxidation product of ustic acid so formed was isolated in good yield as a colourless crystalline substance, $C_{11}H_{10}O_7.H_2O$, which may be described as hydrated dehydro-ustic acid or, as will be shown below, more conveniently as *dehydro-ustic acid*. This compound, which has the properties of a hydrated $\alpha\beta$-diketone, proved to be more amenable to treatment than ustic acid itself so that most of the structural investigations were carried out on it. Dehydro-ustic acid gives the same intense purple colour with aqueous ferric chloride as does ustic acid, but, unlike ustic acid, very quickly gives a precipitate with Brady's reagent and, of course, no longer reduces copper salts. Its aqueous solution at neutral or alkaline reactions is bright yellow in colour, the colour being discharged on acidification. On heating at its melting point, 124–125°, it loses a molecule of water and the resulting clear yellow gum, which does not recrystallize on standing, may be crystallized from undried benzene with the reformation of the colourless hydrated form, but we have not succeeded in obtaining crystalline anhydrous dehydro-ustic acid. However, on methylation of dehydro-ustic acid with dimethyl sulphate and anhydrous potassium carbonate in acetone, there is formed the *methyl ester* of *dimethyldehydro-ustic acid*, $C_{11}H_7O_4(OCH_3)_3$, in lemon-yellow crystals, m.p. 119–120°, which recrystallize on cooling. This substance, on hydrolysis with dilute acid, yields *dimethyldehydro-ustic acid*, $C_{11}H_8O_5(OCH_3)_2$, in colourless prisms from water, m.p. 152–153°, which also recrystallize on cooling and remelt at the same temperature. It will be seen that neither of these methyl derivatives contains the molecule of water which is lost on heating hydrated dehydro-ustic acid. Neither of the methyl derivatives gives any colour with ferric chloride.

The precipitate formed on the addition of Brady's reagent to an aqueous solution of dehydro-ustic acid consists essentially of *dehydro-ustic acid mono-2:4-dinitrophenylhydrazone*,

$$C_{17}H_{14}O_{10}N_4, [C_{11}H_{10}O_6:N.NH.C_6H_3(NO_2)_2],$$

which was obtained as yellow prisms from ethyl acetate-benzene. On heating dehydro-ustic acid with *o*-phenylenediamine in ethanol it gives a crystalline *quinoxaline*,

$$C_{17}H_{14}O_5N_2, (C_{11}H_{10}O_5(N_2C_6H_4)),$$

which is readily soluble in cold aqueous sodium bicarbonate and in 2N-hydrochloric acid.

Degradation experiments with dehydro-ustic acid led to the following results:

(1) The dimethyl ether of dehydro-ustic acid, on oxidation with hot alkaline potassium permanganate, yielded 3:4:6-trimethoxyphthalic acid, identical with a synthetic specimen prepared by Bargellini's (1914) method.

(2) On oxidation with alkaline hydrogen peroxide at room temperature, dehydro-ustic acid gave one molecule of a volatile acid, consisting essentially of acetic acid, and an 80% yield of a crystalline substance which is the unknown *4:6-dihydroxy-3-methoxyphthalic acid*. This substance, on methylation with dimethyl sulphate and potassium carbonate in acetone, also yielded 3:4:6-trimethoxyphthalic acid.

(3) On heating with copper chromite in quinoline, dehydro-ustic acid readily gave one molecule of carbon dioxide and, in fairly good yield, crystalline *decarboxydehydro-ustic acid*, $C_{10}H_{10}O_5$. The same products are formed when dehydro-ustic acid is heated with 2N-sulphuric acid, though the reaction is slow and incomplete (48% carbon dioxide after 6·7 hr. boiling). Decarboxydehydro-ustic acid forms

yellow prisms which do not contain the molecule of water present in dehydro-ustic acid. It is not acidic and cannot be extracted from ethyl acetate solution with aqueous sodium bicarbonate. Its aqueous solution gives an immediate yellow precipitate with Brady's reagent, as does dehydro-ustic acid itself, but, in contrast to the latter which gives an intense stable purple colour with ferric chloride, decarboxydehydro-ustic acid gives with this reagent a blue colour changing rapidly to brown and finally to green. Decarboxydehydro-ustic acid is believed to be 3:5-*dihydroxy-2-methoxyphenyl methyl diketone*.

(4) On oxidation with alkaline hydrogen peroxide, decarboxydehydro-ustic acid gave, in good yield, a crystalline oxidation product which was identified as the previously unknown 3:5-*dihydroxy-2-methoxybenzoic acid*, as follows. (*a*) On methylation it gave 2:3:5-trimethoxybenzoic acid identical with a synthetic specimen. (*b*) On ethylation it gave 3:5-*diethoxy-2-methoxybenzoic acid* identical with a synthetic specimen.

Methods are given in the experimental part of this paper for the synthesis of 2:3:5-trihydroxybenzoic acid, 2:3:5-trimethoxybenzoic acid, 3:5-*diethoxy-2-hydroxybenzoic acid* and 3:5-*diethoxy-2-methoxybenzoic acid*.

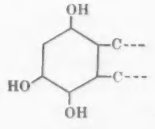
DISCUSSION

Structure of dehydro-ustic acid (VI) and ustic acid (X)

The molecular structure (VI) which we propose for dehydro-ustic acid was deduced from the following experimentally established facts:

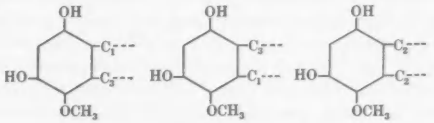
(1) Ustic acid and dehydro-ustic acid each contain one methoxyl group.

(2) Dehydro-ustic acid dimethyl ether, on oxidation with alkaline potassium permanganate yields 3:4:6-trimethoxyphthalic acid. Hence it follows that dehydro-ustic acid must contain the skeleton structure (I), in which one of the hydroxyl groups is methylated.



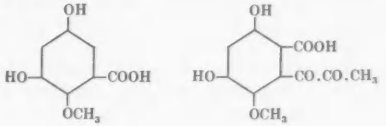
(I)

(3) Anhydro-ustic acid, on treatment in the cold with aqueous sodium hydroxide, gives a small yield of a monomethyl ether of 3:4:6-trihydroxyphthalic acid which, as will be shown later, is 4:6-dihydroxy-3-methoxyphthalic acid (VII). The same compound is given in 80 % of the theoretical yield, together with one molecule of a volatile acid which is almost entirely acetic acid, when dehydro-ustic acid is oxidized with alkaline hydrogen peroxide. Hence structure (I) may be expanded to either structures (II) or (III), since the only other alternative, structure (IV), could not yield both a substituted phthalic acid and acetic acid each in yields approaching the theoretical.



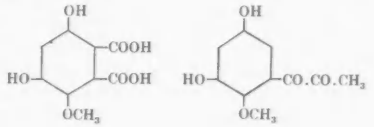
(II) (III) (IV)

(4) A decision between (II) and (III) was made as follows. Dehydro-ustic acid, $C_{11}H_{10}O_7$, on heating with copper chromite and quinoline loses one molecule of carbon dioxide and gives a fairly good yield of decarboxydehydro-ustic acid, $C_{10}H_{10}O_5$, which is a neutral diketone. Decarboxydehydro-ustic acid, on oxidation with alkaline hydrogen peroxide, gives one molecule of acetic acid and, in good yield, 3:5-dihydroxy-2-methoxybenzoic acid, structure (V). Hence dehydro-ustic acid must have the skeleton structure (II), in which the C_3 side chain must be unbranched because of the formation of one molecule of



(V) (VI)

acetic acid on hydrogen peroxide oxidation. We therefore propose structure (VI) for anhydrous dehydro-ustic acid. Since structure (V) was confirmed by synthesis it follows that the monomethyl ether of 3:4:6-trihydroxyphthalic acid described in paragraph (3) above must have structure (VII), and, if structure (VI) is accepted for dehydro-ustic acid, then decarboxydehydro-ustic acid must have structure (VIII).

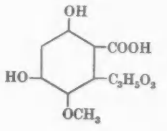


(VII) (VIII)

The fact that dehydro-ustic acid (VI) has been obtained crystalline only as the compound $C_{11}H_{12}O_8$ may be explained by assuming that this formula is more correctly written as $C_{11}H_{10}O_7.H_2O$ since one molecule of water is readily lost on heating and the resulting anhydrous form is a yellow gum which, on recrystallization, is rehydrated to the colourless, crystalline form. Further, all the derivatives of dehydro-ustic acid, i.e. its dimethyl ether, dimethyl

ether methyl ester, mono-2:4-dinitrophenylhydrazone and quinoxaline are derivatives of the anhydrous form $C_{11}H_{10}O_7$.

(5) Since dehydro-ustic acid, $C_{11}H_{10}O_7$, is formed in almost quantitative yield by treating ustic acid, $C_{11}H_{12}O_7$, with cold neutral copper sulphate solution it is reasonable to assume that, under these very mild conditions, no deep-seated change takes place. This reaction involves the loss of two hydrogen atoms by ustic acid and, bearing in mind the similarities and differences in the properties of ustic acid and dehydro-ustic acid already described, is readily explained by postulating that the $—CO.CO.CH_3$ or $—C_3H_3O_2$ side chain in dehydro-ustic acid arises from a similarly placed $—C_3H_5O_2$ side chain in ustic acid. Hence we propose structure (IX) for ustic acid and it now remains to discuss the nature of this $—C_3H_5O_2$ side chain.



(IX)

An unbranched side chain $—C_3H_5O_2$ derived from $—CO.CO.CH_3$ by reduction may have one of the following five forms: (*a*) $—CHOH.CO.CH_3$; (*b*) $—CO.CHOH.CH_3$; (*c*) $—C(OH)=C(OH).CH_3$; (*d*) $—CH(—O—)C(OH).CH_3$; (*e*) $—C(OH).CH(—O—).CH_3$, or an equilibrium mixture of two or more of these possible tautomers depending on the conditions obtaining when any particular test is carried out. It must be admitted that none of our experimental evidence enables us to decide clearly between them, but this evidence for and against each form may be summarized as follows:

(i) Ustic acid rapidly reduces aqueous copper acetate in the cold as does ascorbic acid, which has an ene-diol structure as in (*c*), whereas acetoin gives only slight reduction. Fuson and co-workers (1939 *et seq.*), who have isolated both the interconvertible ene-diol and hydroxy-ketone forms of some highly substituted benzoins, found the ene-diol forms to reduce cold copper acetate with the formation of the corresponding diketones analogous to (VIII) whereas the hydroxy-ketone forms did not.

(ii) Ustic acid, unlike ascorbic acid, does not reduce 2:6-dichlorophenolindophenol. Acetoin gives slight reduction on the alkaline side, but not on the acid side.

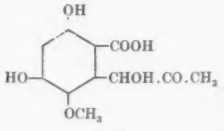
(iii) Neither ustic acid, acetoin nor ascorbic acid gives an immediate precipitate with Brady's reagent. All three compounds give flocculent precipitates on standing.

(iv) Ustic acid was recovered unchanged after prolonged treatment with ozone in either aqueous or ethyl acetate solution. Ascorbic acid, on the other hand, is oxidized rapidly in aqueous acetic acid by ozone to dehydro-ascorbic acid and finally to oxalic acid (Herbert, Hirst, Percival, Reynolds & Smith, 1933).

(v) Ustic acid, in pyridine solution, gave 3·84 active hydrogen atoms in a Zerewitinoff estimation. Structure (IX), with either of the hydroxy-ketone side chains (*a*) or (*b*) or either of the epoxy side chains (*d*) or (*e*), would be expected to give four active hydrogen atoms. If the side chain were the ene-diol form (*c*) five active hydrogen atoms would be expected.

(vi) Ustic acid is optically inactive. This fact might appear to favour the ene-diol form (*c*), which is the only form not containing an asymmetric carbon atom, were it not for the fact that the acetoin isolated from certain bacterial fermentations is also practically optically inactive.

We therefore propose structure (X) to illustrate many of the properties of ustic acid. This is done with the realization that the reactions of ustic acid can be equally well accounted for by postulating the side chain (*b*) $—CO.CHOH.CH_3$, and that some of them

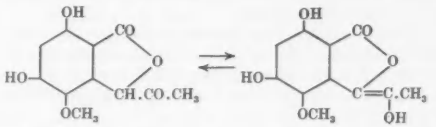


(X)

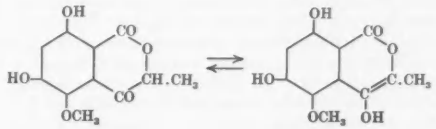
are better represented by the ene-diol form (*c*) $—C(OH)=C(OH).CH_3$. The epoxy side chains (*d*) and (*e*) can also not be excluded if the findings of Kling (1905), that acetoin may exist mainly in the form $CH_3—C(OH)(—O—)CH.CH_3$, be taken to apply to ustic acid.

It was mentioned earlier that ustic acid, when boiled with 2N-sulphuric acid, loses one molecule of water, with the formation of anhydro-ustic acid. Attempts to reconvert this substance into ustic acid, by treatment with cold sodium hydroxide, failed owing to deep-seated decomposition. Further, attempts to methylate or acetylate ustic acid, or to prepare its 2:4-dinitrophenylhydrazone led to the formation of the trimethyl or triacetyl derivatives of anhydro-ustic acid or to its mono-2:4-dinitrophenylhydrazone, all of which substances were also readily obtainable directly from anhydro-ustic acid itself. We believe that anhydro-ustic acid has either structure (XI) or (XII), with a slight preference for (XII), since the *iso*coumarin structure appears to be more stable than the phthalide structure (Bain, Perkin & Robinson, 1914).

Either (XI) or (XII) offers a logical explanation for the loss of one molecule of water in the formation of anhydro-ustic acid and for the marked differences in its properties from those of ustic acid. They also explain the formation of a neutral trimethyl ether or a neutral triacetate in their enol forms (XI*b* or XII*b*) and the formation of a mono-2:4-dinitrophenylhydrazone in their ketone forms (XI*a* or XII*a*).

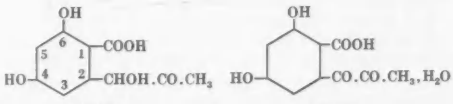


(XI *a*) (XI *b*)



(XII *a*) (XII *b*)

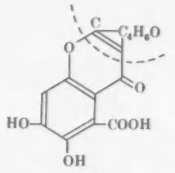
Ustic acid is related structurally to certain other mould metabolic products and to a group of lichen acids. Two unnamed metabolic products of species in the *Penicillium brevi-compactum* Dierckx series have the empirical formulae $C_{10}H_{10}O_6$ and $C_{10}H_{10}O_7$, and the structural formulae (XIII) and (XIV), respectively (Clutterbuck, Oxford, Raistrick & Smith, 1932; Oxford & Raistrick, 1933). It will be seen that ustic acid is the 3-methoxy derivative of



(XIII) (XIV)
$C_{10}H_{10}O_6$ $C_{10}H_{10}O_7$

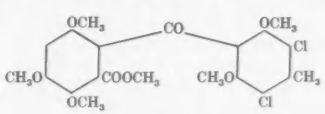
(XIII) and that dehydro-ustic acid is the 3-methoxy derivative of (XIV). The general properties of ustic acid and $C_{10}H_{10}O_6$ are strikingly similar, as are those of dehydro-ustic acid and $C_{10}H_{10}O_7$. So much is this the case that many of the difficulties encountered in elucidating the structures of ustic acid and dehydro-ustic acid had been previously experienced with the acids $C_{10}H_{10}O_6$ and $C_{10}H_{10}O_7$.

Citromycetin, a yellow crystalline metabolite of species formerly included by Wehmer in his genus *Citromyces* but now regarded by Raper & Thom (1949) as belonging to the *Penicillium frequentans* Westling series, was assigned by Hetherington & Raistrick (1931), who first described it, the partial structural formula (XV). When *O*-dimethylcitromycetin methyl ester is hydrolysed with ethanolic potassium hydroxide in nitrogen there is formed in good yield a hydroxydimethoxyphthalic acid, the cleavage being indicated by the dotted line in (XV). We have now shown that methylation of this hydrolytic product yields 3:4:6-trimethoxyphthalic acid identical with that obtained by permanganate oxidation of dehydro-ustic acid dimethyl ether.



(XV)
Citromycetin

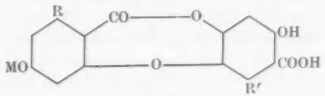
Geodin, $C_{17}H_{12}O_7Cl_2$, and erdin, $C_{16}H_{10}O_7Cl_2$, are metabolic products of *Aspergillus terreus* Thom (Raistrick & Smith, 1936). On methylation with dimethyl sulphate and alkali there is formed from both geodin and erdin the same highly substituted benzoyl benzoic acid shown in structure (XVI) (Calam, Clutterbuck, Oxford & Raistrick, 1947).



(XVI)
Methylated geodin and erdin

This compound can clearly be derived from 3:4:6-trimethoxyphthalic acid and thus links geodin and erdin with ustic acid and citromycetin.

A group of four lichen acids has been investigated by Asahina and co-workers (1933 *et seq.*) who have shown that they all have the general structural formula (XVII), in which R and R′ are carbon chains, and the detailed structures indicated below.

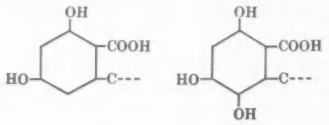


(XVII)

All four acids are built up from the two units (*a*) and (*b*), (*a*) being the skeleton present in the meta-

Physodic acid,	$R=CH_2.CO.C_5H_{11}$,	$R'=C_5H_{11}$,	$M=H$
α-Collatolic acid,	$R=R'=CH_2.CO.C_5H_{11}$,		$M=CH_3$
Alectoronic acid,	$R=R'=CH_2.CO.C_5H_{11}$,		$M=H$
Lobaric acid,	$R=CO.C_4H_9$,	$R'=C_5H_{11}$,	$M=CH_3$

bolites $C_{10}H_{10}O_6$ and $C_{10}H_{10}O_7$ from *Penicillium brevi-compactum*, and (*b*) the skeleton present in ustic acid.



(*a*) (*b*)

A brief account of our work on ustic acid was presented by one of us to the Biochemical Society at its meeting on 18 February 1949 (Stickings, 1949).

EXPERIMENTAL

Cultures

Four different strains of *Aspergillus ustus* (Bainier) Thom & Church were examined in the course of this work. Strains S.A. 906, S.A. 1409 and S.A. 1658 were received from Dr A. L. James, Timber Research Laboratory, Transvaal Chamber of Mines, Exchange Yard, Johannesburg, South Africa. They were isolated from mouldy material occurring in South African mines. Strain BB188 was received from our colleague Mr G. Smith. Their history is as follows. (1) Strain S.A. 906 received 13 June 1947, isolated from decayed flannel used in a mine. (2) Strain S.A. 1409 received 20 October 1947, isolated from decayed ventilation tubing in a mine. (3) Strain S.A. 1658 received 20 October 1947, isolated from a decayed treated flannel bag used in a mine. (4) Strain BB188 isolated by Mr G. Smith 4 August 1943 from a mouldy American wireless set received from the Ministry of Supply.

All four strains were identified as *A. ustus* by Mr G. Smith. Strain S.A. 906 had been determined previously by Dr C. Thom as 'near to *Aspergillus minutus* Abbott'. The latter name is, however, no longer recognized by Thom & Raper (1945), being regarded by them as a synonym of *A. ustus*. Strain S.A. 1658 had been determined previously as *A. ustus* by the Centraalbureau voor Schimmelcultures, Baarn, Holland.

Mr Smith's notes on the four strains of *A. ustus* follow. BB188 is a very typical strain. Colonies spore readily and abundantly, the conidial colour being smoky greyish-brown. Of the three South African strains S.A. 1409 and S.A. 1658 are both somewhat atypical in colony habit and are practically indistinguishable. Colonies are densely floccose, sporing tardily and somewhat sparsely. The colour of the conidial heads is, however, quite typical, smoky greyish-brown. S.A. 906 differs from the other two strains only in sporing even more sparsely. Morphologically all four strains fit well with the description given by Thom & Raper (1945) for *A. ustus*: foot cells very prominent; stalks smooth and brownish; heads at first more or less hemispherical, then irregular both in shape and size; vesicles bearing both metulae and phialides; conidia globose, echinulate, 3·5–4 μ. in diameter.

Cultural conditions and cultural characteristics of the different strains of Aspergillus ustus

Czapek-Dox solution. Glucose, 50 g.; $NaNO_3$, 2 g.; KH_2PO_4, 1 g.; KCl, 0·5 g.; $MgSO_4.7H_2O$, 0·5 g.; $FeSO_4.7H_2O$, 0·01 g.; distilled water, 1 l.

Raulin-Thom solution. Glucose, 75 g.; tartaric acid, 4 g.; ammonium tartrate, 4 g.; $(NH_4)_2HPO_4$, 0·6 g.; $(NH_4)_2SO_4$, 0·25 g.; K_2CO_3, 0·6 g.; $MgCO_3$, 0·4 g.; $FeSO_4.7H_2O$, 0·07 g.; $ZnSO_4.7H_2O$, 0·07 g.; distilled water, 1·5 l.

Portions of these solutions (350 ml.), contained in a series of 1 l. conical flasks plugged with cotton wool, were sterilized and inoculated with one or other of the four strains of *A. ustus* and were incubated in the dark at 24°. Single flasks were harvested after 7, 14 and 21 days' incubation and the culture filtrates from these were examined by Mrs S. Marcus with the following results.

None of them, in either medium and after either 7 or 14 days' incubation, showed any antibacterial activity at a dilution of 1:10 against either the Oxford H strain of *Staphylococcus aureus* or the N.C.T.C. strain 86 of *Escherichia coli*.

None of the Czapek-Dox filtrates of strains S.A. 906, S.A. 1409 or S.A. 1658 gave more than a pale pinkish-buff colour with $FeCl_3$, while those from strain BB188 gave a moderate purplish-brown colour after 14 and 21 days' incubation.

The Raulin-Thom filtrates of all four strains gave purple to purplish-brown $FeCl_3$ colours varying in intensity from moderate with strain S.A. 1658 to very intense with S.A. 906. Br_2 water was rapidly decolorized by S.A. 906 and BB188 after 21 days' incubation, but little precipitate was formed.

As a result of these findings the relative intensities of the maximum colours produced by the addition of aqueous $FeCl_3$ to 10 ml. of the 21-day Raulin-Thom culture filtrates were compared in a colorimeter. The results obtained and other estimations carried out are summarized in Table 1.

Table 1. *Cultural characteristics of culture filtrates of four strains of* Aspergillus ustus

Strain no.	Relative intensity of $FeCl_3$ colour	pH	Residual glucose by polarimeter (%)
S.A. 906	9	4·1	0·32
S.A. 1409	3	5·1	0·16
S.A. 1658	1	6·1	0·02
BB 188	3	4·1	0·27

Comparison of the relative intensity of $FeCl_3$ colours given by Raulin-Thom culture filtrates of *A. ustus* strain S.A. 906 after 16–39 days' incubation and harvested every 2–3 days showed clearly that the intensity of the $FeCl_3$ colour reaches a maximum after 18–21 days' incubation and thereafter slowly decreases. After 39 days' incubation the pH was 5·5, the glucose had almost completely disappeared and the $FeCl_3$ intensity was about 90% of that at its peak. Strain S.A. 906 was therefore used for the bulk preparation of ustic acid and the cultures were harvested after 21 days' incubation.

Isolation of crude ustic acid from culture filtrates of Aspergillus ustus *strain S.A. 906*

Batches of 100-flask cultures of *A. ustus* strain S.A. 906, grown on Raulin-Thom medium, were harvested after 21 days' incubation at 24°. At the end of this time the upper surface of the mycelium was white and the reverse yellow. The culture fluid was also yellow and each flask contained a small amount of yellow amorphous sediment. The culture fluid was decanted from the mycelium and clarified by

filtration through a pad of kieselguhr on a large filter. The clear yellow filtrate (31 l.) was acidified to congo red with conc. HCl (3 ml./l.). Crude ustic acid was isolated from the acidified filtrate by one of the following methods:

(*a*) It was extracted with ethyl acetate four times, using 1/4 vol. of ethyl acetate for the first extraction and 1/10 vol. for each of the other three extractions. This treatment removed about 85% of the ustic acid, and the residual aqueous layer which was discarded gave a brown $FeCl_3$ reaction. The combined ethyl acetate extracts were evaporated under reduced pressure at 40° leaving a brown viscous gum (47 g.). This was treated with ether (400 ml.), when an amorphous buff-coloured solid separated and was filtered off (7 g.). It gave no $FeCl_3$ reaction, and has not been investigated further. On removal of the ether there remained a pale-brown viscous gum (40 g.), a portion of which, dissolved in water, gave only a slight immediate precipitate with Brady's reagent (2:4-dinitrophenylhydrazine in 2N-HCl).

(*b*) Charcoal (8 g./l.), previously activated by heating to 135° for 2 hr., was added to the acidified filtrate. The suspension was stirred for some hours and then allowed to settle. The charcoal was separated by filtration, washed with water and dried in a desiccator. The filtrate, which gave little colour with $FeCl_3$, was discarded. The dried charcoal was extracted with dry ether in a long wide glass column, by percolation under gravity, during several days. On removal of the ether from the extract there remained a pale-brown gum (22 g.), a portion of which gave an immediate copious precipitate with Brady's reagent.

Purification of crude ustic acid

The gums obtained by either of the methods (*a*) or (*b*) proved difficult to crystallize and were finally purified by taking advantage of the fact that ustic acid forms a K salt which is only slightly soluble in methanol. The gums obtained from each batch of 100 flasks either by method (*a*) or (*b*) were separately dissolved in cold methanol (2 ml./g.), and a 25% solution of anhydrous potassium acetate in methanol (10 ml./g.) was added. Crystals began to separate at once from the gums prepared by either method. After holding for 2 days in a refrigerator, the crystals were collected by filtration, washed with methanol and ether, and dried. Average yield of K salt from method (*a*) 21–24 g. of buff-coloured prisms giving only a slight immediate precipitate with Brady's reagent; by method (*b*) 11–12 g. of yellow crystals giving an immediate copious precipitate with Brady's reagent.

The crude K salt (24 g.) from method (*a*) was dissolved in a minimum of water, acidified to congo red with HCl, and the solution was extracted twice with an equal volume of ethyl acetate. The combined extracts, after washing with a little water and re-extraction of the wash water with ethyl acetate, were evaporated under reduced pressure to about 100 ml., and boiling benzene (500 ml.) was added. On cooling and allowing to stand in the refrigerator for 2 days, 15·0 g. of pale-brown crystals of ustic acid, m.p. 166–167° (decomp.), were obtained. A further 2·5 g. of less pure crystalline material was obtained by evaporating the filtrate and treating with light petroleum. The recovery yield from the K salt was 85%. The final mother liquors, on evaporation, yielded a gum (1·3 g.) which could not be crystallized.

The crude K salt (11–12 g.) from method (*b*), when treated similarly, gave a 60% recovery yield of ustic acid, m.p. 166–167° (decomp.). From the mother liquors a small amount of dehydro-ustic acid, m.p. 124° (see p. 62), separated on standing. This substance, which is doubtless an artifact arising by oxidation of ustic acid in the charcoal extraction method (*b*), accounts for the formation of an immediate precipitate with Brady's reagent from the K salt.

The crystalline ustic acid prepared by either method (*a*) or (*b*) was recrystallized by dissolving in boiling ethyl acetate (5 ml./g.) and adding boiling benzene (25 ml./g.) with the addition of charcoal. On cooling the filtrate, ustic acid separated in rosettes of colourless rhombic prisms, m.p. 167° (decomp.). The colour of the crude ustic acid can also be removed by passing an ethyl acetate solution of it through a short alumina column. By repeated recrystallization and purification the melting point of ustic acid was raised to 169–170° (decomp.).

The average overall yield of crystalline ustic acid obtained by method (*a*) from a number of batches of cultures of *A. ustus* strain S.A. 906 was about 0·5 g./l. of culture filtrate. By method (*b*) the yield was 0·2 g./l. of culture filtrate.

As will be shown later, ustic acid contains a methoxyl group so that there is a possibility that this group might have been introduced by the use of methanol in the purification procedure described above. This possibility was disproved as follows. In one of the isolation experiments using the charcoal method (*b*) a portion of the dried charcoal was eluted with $CHCl_3$ instead of ether. The $CHCl_3$ eluate was evaporated to dryness, extracted with ether and filtered. On removal of the solvent from the ether filtrate, a gum (1·75 g.) remained. This was dissolved in acetone (2 ml.) and treated with boiling $CHCl_3$ (100 ml.). Crystalline ustic acid (0·39 g.) separated, m.p. 166–167° (decomp.) not depressed on admixture with ustic acid, m.p. 167–168° (decomp.) prepared via the K salt from methanol solution.

Isolation of ustic acid from culture filtrates of Aspergillus ustus *strains S.A. 1409, S.A. 1658 and BB 188*

Ustic acid was also obtained, in much smaller amounts, from culture filtrates of the strains S.A. 1409, S.A. 1658 and BB188. Growth on Raulin-Thom medium followed a similar course to that of strain S.A. 906, the maximum intensity of the $FeCl_3$ reaction being attained after 21, 28 and 21 days, respectively. Flasks were harvested and treated

Table 2. *The isolation of ustic acid from four strains of* Aspergillus ustus

Strain	Incubation period (days)	Residual glucose by polarimeter (%)	pH	Metabolism solution (g./l.) Crude extract	Ether-insoluble	K salt	Crystalline ustic acid	M.p. of ustic acid (°)
S.A. 906	21	0·32	4·1	1·5	0·4	0·75	0·5	166–167
S.A. 1409	24	0·08	5·3	0·56	0·15	0·12	0·05	160–165
S.A. 1658	24	0·00	6·9	0·21	0·04	0·01	0·001	164–166
BB188	21	0·43	4·5	0·46	0·12	0·10	0·04	167–168

by method (*a*), with the results shown in Table 2. The K salts from these three strains all gave an immediate precipitate with Brady's reagent, suggesting the presence of dehydro-ustic acid. The ustic acid obtained was identified by mixed melting point and by comparison of properties with the material from S.A. 906.

Although strain BB188 gave a purplish $FeCl_3$ reaction when grown on Czapek-Dox medium, no ustic acid seemed to be formed. Growth followed a different course, the most striking characteristic being the formation of orange pigments, which are being further investigated. The gummy residue from ethyl acetate extraction of the metabolism solution (after removal of the pigment) gave a red colour with $FeCl_3$, but did not reduce cold Fehling's solution, and treatment with 25% methanolic potassium acetate failed to give any K salt.

General properties of ustic acid

Ustic acid crystallizes in colourless rhombic prisms, m.p. 169–170° with gentle decomposition, a gas being evolved and a straw-coloured liquid remaining which does not recrystallize on cooling. It does not contain N, S, or Cl. (Found: C, 51·8, 51·5; H, 4·7, 4·8; OCH_3, 12·0; *C*-CH_3, 8·7% = 1·47 *C*-CH_3 groups*; active H atoms (in pyridine), 3·8. $C_{11}H_{12}O_7$ requires: C, 51·6; H, 4·7; 1OCH_3, 12·1; 1*C*-CH_3, 5·9%.) Ustic acid has no optical activity. It dissolves readily in water, ethanol, methanol, acetone and dioxan, less readily in ethyl acetate (about 10 vol./g.) and with difficulty in ether. It is almost insoluble in benzene, $CHCl_3$, CCl_4 or light petroleum. An aqueous solution of ustic acid has the following properties: (1) It is colourless and acid in reaction (pH of 0·05 N-solution, 3·4), and becomes only faintly coloured on the addition of alkali. (2) Titration with 0·1 N-NaOH, using phenolphthalein as indicator, gives a very indefinite end point. The equivalent lies between 128 and 256, suggesting one strong and one or more weak acidic groups. (3) It shows a pale blue fluorescence in ultraviolet light. An acetone solution gives a pale greenish-blue fluorescence. (4) Aqueous $FeCl_3$ gives an intense purple colour which is stable for many hours. In ethanolic solution the colour is much bluer. (5) On gradual addition of Br_2 water the latter is decolorized. No colour develops until excess of Br_2 is present and no turbidity or precipitate appears. (6) With Brady's reagent the solution remains clear for 2 hr. Over a period of 5 days fine yellow needles are slowly deposited (see p. 61). (7) The addition, at room temperature, of Fehling's solution, or aqueous $CuSO_4$ in neutral solution, or copper acetate solution, results in rapid reduction to cuprous oxide and the formation of dehydro-ustic acid (see p. 62).

Potassium ustate. Pure ustic acid (1·0 g.) dissolved in methanol (2 ml.) was treated with 25% methanolic potassium acetate solution (10 ml.). After standing in the refrigerator overnight, the slightly yellow crystals were collected (1·14 g., theoretical yield) and recrystallized from boiling water, from which colourless hexagonal plates of potassium ustate separated (0·43 g.). An aqueous solution of these crystals had a pH of 7·0. Potassium ustate (0·0485 g.), after ashing with conc. H_2SO_4, yields 0·0144 g. of anhydrous K_2SO_4, corresponding to an equivalent of 255 for ustic acid; $C_{11}H_{11}O_7K$ requires equiv. 256.

Anhydro-ustic acid

A solution of ustic acid (0·4842 g.) in 2 N-H_2SO_4 (20 ml.) was boiled under reflux, and a slow stream of N_2 was passed through the reaction mixture. The issuing gas was scrubbed, first through a bubbler containing Brady's reagent, and then through one containing 0·2 N-aqueous $Ba(OH)_2$. The reaction was complete in 1 hr. No precipitate appeared in the Brady's reagent, and only 8% of the theoretical amount for 1 mol. of CO_2 was absorbed in the baryta.

The straw-coloured reaction mixture was diluted to 100 ml., partly neutralized with 2 N-NaOH to pH 2 and then extracted twice with an equal volume of ethyl acetate. The combined extracts were washed once with water (10 ml.) and evaporated to dryness. The residual, almost colourless, crystalline solid (0·41 g.) was crystallized first from ethyl acetate (8 ml.) and then from benzene (25 ml.). *Anhydro-ustic acid* was thus obtained as colourless needles, m.p. 145–159°. The melting point is very indefinite, the crystals melting to a pale-yellow liquid which does not readily solidify on cooling. (Found: C, 55·3, H, 4·4; OCH_3, 12·9, 12·9. $C_{11}H_{10}O_6$ requires: C, 55·5; H, 4·2; 1OCH_3, 13·0%.)

Anhydro-ustic acid is somewhat soluble in water from which it crystallizes in colourless rods. Its aqueous solution gives a number of reactions which are markedly different from those given by the parent substance. (1) It becomes yellow on the addition of dil. NaOH. (2) Aqueous $FeCl_3$ gives a purple colour which is much fainter than that given by ustic acid and which, on standing, becomes deeper and redder. In ethanolic solution the colour is pale green, changing to yellow on dilution with water. (3) Brady's reagent gives an immediate yellow precipitate. (4) It does not reduce Fehling's solution in the cold. (5) It can be extracted by solvents from a solution in aqueous $NaHCO_3$.

Alkaline hydrolysis of anhydro-ustic acid. A solution of anhydro-ustic acid (0·1 g.) in 2 N-NaOH (10 ml.) was held at room temperature for 3 days. The solution, initially yellow, turned green after 1 hr. and remained that colour. It no longer gave a precipitate with Brady's reagent, nor did it reduce cold Fehling's solution. The solution was acidified to congo red and extracted with ethyl acetate (20 ml.). The extract was evaporated leaving a red gum (0·075 g.) which was dissolved in ethyl acetate (2 ml.), and benzene (20 ml.) was added. Tiny pink nodules separated (0·03 g.), m.p. 191° (decomp.) with the formation of a crystalline sublimate, m.p. 174–177°. The melting point of the substance was not depressed on admixture with 4:6-dihydroxy-3-methoxyphthalic acid (see p. 63). It was recrystallized from ethyl acetate (1 ml.) and benzene (5 ml.) with charcoal, the filtrate being diluted with an equal volume of light petroleum b.p. 60–80°. The product separated as colourless prisms (0·01 g.), m.p. 194–195° (decomp.). (Found: C, 47·5; H, 3·8. Calc. for $C_9H_8O_7$: C, 47·4; H, 3·5%.) An aqueous solution of the compound gave a pure purple colour with aqueous $FeCl_3$, and did not give a precipitate with Brady's reagent.

Ustic acid itself, hydrolysed in the same way, gave a deep-red solution no longer reducing Fehling's solution, but giving an immediate precipitate with Brady's reagent. Extraction with ethyl acetate, after acidification, yielded a gum (0·085 g.) an aqueous solution of which gave an intense purple colour with aqueous $FeCl_3$. No pure substance was, however, isolated from the gum.

* Ustic acid and a number of its derivatives and breakdown products give abnormally high *C*-CH_3 values, cf. the two acetates of anhydro-ustic acid (p. 61); dehydro-ustic acid (p. 62); and, particularly, 4:6-dihydroxy-3-methoxyphthalic acid (p. 63) and 3:4:6-trimethoxyphthalic acid (p. 64) neither of which contains a *C*-CH_3 group.

DERIVATIVES OF USTIC ACID AND ANHYDRO-USTIC ACID

Products of methylation

(*a*) *With diazomethane.* Ustic acid, treated in ethereal solution with ethereal diazomethane for 2 days, yielded a gum from which no pure substance could be isolated. An aqueous solution of the gum no longer reduced cold Fehling's solution, nor did it give any colour with $FeCl_3$. On the other hand, Brady's reagent yielded an immediate precipitate.

(*b*) *With dimethyl sulphate and aqueous* NaOH. Ustic acid gave very similar results to those obtained in method (*a*).

(*c*) *With dimethyl sulphate and* K_2CO_3 *in acetone.* Ustic acid (0·20 g.), anhydrous K_2CO_3 (1 g.), dimethyl sulphate (1 ml.) and dry acetone (20 ml.) were heated under reflux for 1·5 hr. Volatile solvents were removed on the water pump and the residue was vigorously shaken with water (20 ml.). A solid (0·075 g.) separated. It was twice crystallized from methanol yielding colourless rhombic plates of *trimethylanhydro-ustic acid*, m.p. 171–172·5°. (Found: C, 60·3; H, 6·0; OCH_3, 43·5. $C_{14}H_{16}O_6$ requires: C, 60·0; H, 5·8; $4OCH_3$, 44·3%.) Trimethylanhydro-ustic acid is neutral in reaction and insoluble in cold NaOH.

From the aqueous filtrate from the crude trimethylanhydro-ustic acid there was isolated a small quantity of 3:4:6-trimethoxyphthalic anhydride. In repeat experiments this product was not obtained, while the yield of trimethylanhydro-ustic acid was low and variable and purification difficult. In one case a small amount of dimethyldehydro-ustic acid (see p. 62) was isolated. The nature of these two by-products suggests that oxidation is liable to occur at some stage in the reaction.

Anhydro-ustic acid (0·10 g.), methylated as described above for ustic acid, gave, in about the same yield, trimethylanhydro-ustic acid, m.p. 170–172°; it did not depress the melting point of the corresponding product from ustic acid itself.

(*d*) *With dimethyl sulphate and* K_2CO_3 *in methanol.* Ustic acid (0·20 g.), anhydrous K_2CO_3 (1 g.), dimethyl sulphate (1 ml.) and dry methanol (20 ml.) were heated under reflux for 1·5 hr., yielding finally a yellow solution. The methanol was removed and water (20 ml.) was added. The resulting clear yellow solution was extracted twice with an equal volume of ethyl acetate which, on evaporation, left a negligible residue. The aqueous solution was acidified to congo red and re-extracted twice with an equal volume of ethyl acetate. On removal of the solvent, a colourless gum (0·19 g.) remained and crystallized partially on standing. Treatment of the gum with dry ether yielded a crystalline powder (0·075 g.), m.p. 155–162° (slight decomp.). After three crystallizations from water, *ustic acid 4-methyl ether* separated as large, almost colourless, rhombic prisms (0·01 g.), m.p. 181–182° with slight decomposition to a yellow liquid. (Found: C, 52·9; H, 5·1. $C_{12}H_{14}O_7$ requires: C, 53·3; H, 5·2%.) An aqueous solution of ustic acid 4-methyl ether gives a deep-purple colour with $FeCl_3$, reduces cold aqueous copper acetate and gives a precipitate with Brady's reagent only after some hours.

Products of acetylation

(*a*) *With acetic anhydride and sodium acetate.* Ustic acid (0·1 g.), anhydrous sodium acetate (0·1 g.) and acetic anhydride (1 ml.) were mixed and boiled for 1 min. The pale-yellow solution was cooled and poured into water. A gum, which was insoluble in cold NaOH, separated, but no pure substance could be isolated from it.

(*b*) *With acetic anhydride and pyridine.* Ustic acid (0·5 g.) was dissolved in pyridine (6 ml.), and acetic anhydride (5 ml.) was added. The solution was held at room temperature for 6 days and then poured into ice water (100 ml.) when a gum separated. After acidification to congo red the mixture was extracted with benzene. The benzene solution was washed twice with 2N-HCl, then with water, dried over Na_2SO_4 and evaporated. The residual gum (0·71 g.) was redissolved in benzene (50 ml.) and extracted twice with fresh, 5% aqueous $NaHCO_3$. The $NaHCO_3$ extract was rejected. The residual gum (0·63 g.) from the benzene layer was treated with dry ether (20 ml.). It dissolved completely, but crystals separated in the refrigerator, wt. 0·14 g., m.p. 155–158°. These were crystallized three times from methanol yielding *triacetylanhydro-ustic acid* as colourless rhombic prisms, m.p. 159–160°. (Found: C, 56·7; H, 4·4; OCH_3, 8·5, 8·9; *C*-CH_3, 18·5. $C_{11}H_7O_3(OCOCH_3)_3$ requires: C, 56·0; H, 4·4; $1OCH_3$, 8·5; $4C$-CH_3, 16·5 or 18·5% if allowance is made for the high value obtained with ustic acid.) Triacetylanhydro-ustic acid is neutral in reaction and insoluble in water.

The ether filtrate from triacetylanhydro-ustic acid (0·14 g.) was treated with an equal volume of light petroleum and held at 0° for some days. The crystals so obtained (0·18 g.), m.p. 120–124°, were recrystallized from aqueous methanol and then from ethanol. The *compound*, the nature of which is not clear, was thus obtained as colourless rhombic prisms, m.p. 125–126°. (Found: C, 53·7; H, 4·8; OCH_3, 7·6, 7·5; *C*-CH_3, 22·1. $C_{11}H_8O_3(OCOCH_3)_4$ requires: C, 53·8; H, 4·8; $1OCH_3$, 7·3; $5C$-CH_3, 17·7 or 19·4% if allowance is made for the high value obtained with ustic acid.) This compound, which is neutral in reaction and insoluble in water, is very similar in appearance to, though differing greatly in melting point from, triacetylanhydro-ustic acid.

Anhydro-ustic acid (0·2 g.) was dissolved in pyridine (2·5 ml.), and acetic anhydride (2 ml.) was added. The solution was held for several days at room temperature and was then poured into water (50 ml.). The resulting gum soon solidified and was separated by filtration (weight 0·15 g., m.p. 137–150°). Two crystallizations from ethanol yielded triacetylanhydro-ustic acid (0·08 g.), m.p. 160–161·5°, not depressing the melting point of triacetylanhydro-ustic acid prepared from ustic acid itself.

Reaction product with 2:4-*dinitrophenylhydrazine*

Ustic acid (0·20 g.) was dissolved in water (5 ml.), and Brady's reagent, 0·32% 2:4-dinitrophenylhydrazine in 2N-HCl, (150 ml.) was added. The initially clear solution began to cloud after 2 hr., and after 3 days there was copious separation of fluffy balls of yellow needles (0·14 g.). A further crop (0·15 g.) separated during the next 2 days. After two crystallizations from ethyl acetate *anhydro-ustic acid mono-2:4-dinitrophenylhydrazone* was obtained as characteristic hexagonal yellow prisms. (Found: C, 49·1; H, 3·9; N, 12·3; OCH_3, 7·3. $C_{17}H_{14}O_9N_4$ requires: C, 48·8; H, 3·4; N, 13·4; $1OCH_3$, 7·4%.) The substance has no definite melting point. It softens at 140°, partly melts at 150°, partly resets at 160° and finally remelts at 180°; when heated very rapidly it softens at 143° and melts at 152–160° with decomposition. The compound gives a red colour when dissolved in ethanol and treated with a drop of aqueous NaOH (Neuberg's test)

indicating a mono-derivative. It is insoluble in $NaHCO_3$, but dissolves in Na_2CO_3 to give a red colour.

Anhydro-ustic acid (0·10 g.) was dissolved in water (6 ml.), and Brady's reagent (80 ml.) was added. An immediate non-crystalline precipitate was formed. It was separated by filtration, washed, dried (weight 0·185 g.) and crystallized from ethyl acetate. Anhydro-ustic acid mono-2:4-dinitrophenylhydrazone, identical in properties and appearance—characteristic hexagonal yellow prisms—with the product from ustic acid described above, was thus obtained. The dinitrophenylhydrazone from ustic acid and the dinitrophenylhydrazone from anhydro-ustic acid and a mixture of the two, when heated in the same bath, behaved in the same way.

Action of ozone on ustic acid

(*a*) *In water.* Ustic acid (0·22 g.) was dissolved in water (20 ml.) and cooled in ice water. Ozonized oxygen was passed through the solution for 4 hr. At the end of this time the solution still reduced cold Fehling's solution and gave only a slight cloudiness with Brady's reagent. Evaporation of the solution under reduced pressure and titration of the distillate yielded only 5% of the theoretical for the production of 1 mol. of volatile acid. The residue after evaporation was crystallized from ethyl acetate (3 ml.) and benzene (30 ml.) and yielded unchanged ustic acid, m.p. 166° (decomp.), weight 0·155 g. = 70% recovery.

(*b*) *In ethyl acetate.* A solution of ustic acid (0·25 g.) in ethyl acetate (20 ml.) was similarly ozonized for 6 hr. Water (50 ml.) was added and the whole was evaporated to dryness *in vacuo*. Crystallization of the residue yielded unchanged ustic acid, m.p. 167° (decomp.), weight 0·17 g. = 68% recovery.

DEHYDRO-USTIC ACID

Ustic acid (0·255 g., 0·0010 g. mol.) and $CuSO_4.5H_2O$ (0·6 g., 0·0024 g. mol.) were dissolved in cold water (10 ml.). A green solution resulted, but no Cu_2O separated. 0·1 N-NaOH was added from a burette until the solution became permanently neutral (pH 7). A copious precipitate of Cu_2O separated. The amount of NaOH required (50 ml.) is that necessary to neutralize the products of the reaction:

$$C_{11}H_{12}O_7 + 2CuSO_4 + 2H_2O \rightarrow C_{11}H_{12}O_8 + Cu_2O + 2H_2SO_4.$$

The Cu_2O was separated by filtration and the clear filtrate was acidified to congo red and extracted twice with an equal volume of ethyl acetate. The gum (0·24 g.) remaining after removal of the solvent was crystallized from water (1·5 ml.) using charcoal, when *dehydro-ustic acid* separated in colourless diamond-shaped prisms (0·16 g.); a further quantity (0·03 g.) of less pure material was obtained from the liquors; total yield 70%. A second crystallization from water gave the pure acid, m.p. 124–125° with loss of water, the melt not resetting on cooling. The melting point is somewhat dependent on the rate of heating since water is lost slowly even below the melting point. (Found, on material dried *in vacuo* at room temperature: C, 48·3; H, 4·7; OCH_3, 11·4; *C*-CH_3, 6·2; loss at 100°/16 mm., 6·9. $C_{11}H_{12}O_8(C_{11}H_{10}O_7.H_2O)$ requires: C, 48·5; H, 4·4; $1OCH_3$, 11·4; $1C$-CH_3, 5·5; loss of $1H_2O$, 6·6%.) The clear yellow gum which results from the loss of water on heating dehydro-ustic acid does not crystallize on standing. However, when dissolved in boiling undried benzene, crystals of dehydro-ustic acid separate, m.p. 123–125° with loss of water, the melting point being unchanged on admixture with hydrated dehydro-ustic acid.

Dehydro-ustic acid dissolves fairly readily in ethyl acetate and water. It is slightly soluble in hot benzene from which it can be crystallized, and is almost insoluble in light petroleum. An aqueous solution of dehydro-ustic acid has the following properties: (1) At neutral or alkaline pH values it is yellow, the colour being discharged on acidification. (2) It shows no fluorescence in ultraviolet light, nor does an acetone solution of dehydro-ustic acid. (3) Aqueous $FeCl_3$ gives an intense purple colour indistinguishable from that given by ustic acid. (4) On gradual addition of bromine water the latter is decolorized, but a transient cloudiness is produced, disappearing after a few seconds. At no time is a permanent precipitate observed. (5) With Brady's reagent an immediate cloudiness is produced, followed shortly by the formation of a copious flocculent precipitate. (6) It does not reduce Fehling's solution at room temperature or aqueous $CuSO_4$ or copper acetate solution.

Derivatives of dehydro-ustic acid

Methyl derivatives. A mixture of hydrated dehydro-ustic acid (0·71 g.), dimethyl sulphate (3 ml.), anhydrous K_2CO_3 (3 g.) and dry acetone (50 ml.) was refluxed for 1·5 hr. Volatile solvents were removed on the water pump, water (50 ml.) was added to the residue, and the alkaline mixture was shaken vigorously for 20 min. when the oil which had separated crystallized to a yellow solid which was separated by filtration (wt. 0·52 g., m.p. 118–120°). This *methyl dimethyldehydro-ustate* crystallized from benzene-light petroleum in pale-yellow rectangular prisms. For analysis the substance was sublimed at 110–115° in a high vacuum, the lemon-yellow sublimate melting at 119–120° without decomposition and recrystallizing on cooling. (Found: C, 57·1; H, 5·5; OCH_3, 41·6. $C_{14}H_{16}O_7$ requires: C, 56·8; H, 5·4; $4OCH_3$, 41·9%.) Methyl dimethyldehydro-ustate gives no colour with ethanolic $FeCl_3$, but gives an immediate yellow precipitate with Brady's reagent in aqueous ethanol.

The alkaline aqueous filtrate from the ester, after standing at room temperature for 24 hr., was extracted twice with an equal volume of ether, the extract being rejected. The aqueous layer was then acidified to congo red and extracted three times with an equal volume of ethyl acetate. On removal of the solvent a solid (0·18 g.), m.p. 148–151°, remained. This was crystallized three times from water from which *dimethyldehydro-ustic acid* separated as long colourless rhombic prisms or needles, m.p. 152–153° without decomposition and resetting on cooling. (Found: C, 54·8; H, 5·3; OCH_3, 32·6. $C_{12}H_{14}O_7$ requires: C, 55·3; H, 5·0; $3OCH_3$, 33·0%.) Dimethyldehydro-ustic acid is soluble in cold aqueous $NaHCO_3$. It gives no colour with aqueous $FeCl_3$ and gives an immediate precipitate with Brady's reagent. Alkaline hydrolysis of methyl dimethyldehydro-ustic acid leads to breakdown. Acid hydrolysis was more successful. A mixture of the ester (0·05 g.) and 2 N-HCl (1 ml.) was boiled for 5 min. after the solid had dissolved. On cooling, colourless crystals (0·045 g.) separated, m.p. 150–152° unchanged on admixture with dimethyldehydro-ustic acid.

Mono-2:4-dinitrophenylhydrazone. Dehydro-ustic acid (0·2 g.) was dissolved in water (5 ml.), and Brady's reagent (150 ml.) was added. The mixture was held at room temperature for 3 days when the initial flocculent precipitate had settled to a crystalline solid which was collected (0·32 g.). It was recrystallized by dissolving in hot ethyl acetate (2 ml.) and adding benzene (10 ml.). *Dehydro-ustic acid mono-2:4-dinitrophenylhydrazone* separated as yellow prisms (0·26 g.).

When heated slowly it softens at 155° and decomposes slightly without discoloration then resets to a yellow solid and remelts at 222–227° with vigorous decomposition and darkening; if inserted in a bath at 200° and heated, it decomposes vigorously and sets at once, then decomposes at 230° with darkening. Further crystallization from ethyl acetate-benzene or from ethyl acetate-light petroleum did not change this behaviour. (Found, on material crystallized from ethyl acetate-light petroleum and dried at 100°/16 mm.: C, 47·7; H, 3·8; N, 13·2; OCH_3, 7·1; loss in weight on drying, 4·4. $C_{17}H_{14}O_{10}N_4$ requires: C, 46·9; H, 3·2; N, 12·9; $1OCH_3$, 7·1; loss of $1H_2O$ from $C_{17}H_{14}O_{10}N_4.H_2O$, 4·0%.) The compound gives a red colour with Neuberg's reagent, indicating a mono-derivative. It is soluble in $NaHCO_3$ to give a yellow solution, and in Na_2CO_3 to give a red solution.

Quinoxaline. Dehydro-ustic acid (0·27 g.) and *o*-phenylenediamine hydrochloride (0·20 g., 10% excess) in ethanol (10 ml.) was refluxed for 30 min. The green solution was cooled and poured into water (50 ml.). The almost colourless crystalline solid which separated (weight 0·30 g., m.p. 216° (decomp.)) was recrystallized three times from 30 to 40% aqueous ethanol, using charcoal. The *quinoxaline* forms slightly yellow needles or plates, m.p. 226–227° with vigorous gas evolution. The air-dried material contains water and ethanol. (Found, on material dried at 100°/16 mm.: C, 62·3; H, 4·3; N, 8·8; OCH_3, 9·6. $C_{17}H_{14}O_5N_2$, the quinoxaline from $C_{11}H_{10}O_7$, requires: C, 62·6; H, 4·3; N, 8·6; $1OCH_3$, 9·5%.)

The quinoxaline is only slightly soluble in boiling water, but dissolves in cold $NaHCO_3$ and in 2N-HCl. In ethanolic solution it gives a bluish-grey colour with $FeCl_3$, which changes to purple on dilution with water. Solutions in ethanol or benzene do not fluoresce in ultraviolet light. The brown solution in cold conc. H_2SO_4 turns to deep blue on warming.

Oxidation of dimethyldehydro-ustic acid by alkaline $KMnO_4$

Dimethyldehydro-ustic acid (0·24 g.) was dissolved in a small excess of aqueous Na_2CO_3 and boiled under reflux with 0·05M-$KMnO_4$ (100 ml.), further 50 ml. portions being added until the red colour persisted for 30 min. (total volume 350 ml.). The mixture was cooled, decolorized with $NaHSO_3$, acidified and extracted twice with an equal volume of ethyl acetate. The extracts were washed with water (20 ml.) and evaporated to give a crude solid residue (0·13 g.). After two recrystallizations from water, 3:4:6-trimethoxyphthalic acid (87·5 mg.) separated as colourless rhombic tablets, m.p. 210–211° after softening at 180°. If placed in a bath heated above 180° the crystals melt at once evolving water, then reset, and remelt at 210–211°. (Found, on air-dried material: loss of weight at 100°/16 mm., 6·8. Calc. for $C_{11}H_{12}O_7$ H_2O: loss of $1H_2O$, 6·6%. Found, on material dried at 100°/16 mm. C, 51·7, 51·6; H, 4·7, 4·6; OCH_3, 37·0, 36·3. Calc. for $C_{11}H_{12}O_7$: C, 51·6; H, 4·7; $3OCH_3$, 36·3%.) The acid gives no colour with $FeCl_3$.

The above 3:4:6-trimethoxyphthalic acid (27 mg.) was heated in an open test tube in an oil bath until the bath temperature reached 220°. At this point yellowish leaflets began to appear as a sublimate. The tube was cooled and the whole contents were crystallized from benzene (5 ml.). The solution, which showed a strong blue-purple fluorescence, deposited short stout prisms of 3:4:6-trimethoxyphthalic anhydride (weight 18 mg., m.p. 212–213°). (Found: C, 55·5, 55·4; H, 4·2, 4·1; OCH_3, 38·8. Calc. for $C_{11}H_{10}O_6$: C, 55·5; H, 4·2; $3OCH_3$, 39·1%.)

Synthetic 3:4:6-trimethoxyphthalic acid and 3:4:6-trimethoxyphthalic anhydride were prepared according to the method of Bargellini (1914). They were identical in appearance and properties with the oxidation products recorded above. Synthetic 3:4:6-trimethoxyphthalic acid melted at 209–210° and did not depress the melting point of the oxidation acid. The synthetic anhydride melted at 209–210°; mixed with the oxidation anhydride it melted at 209–211°.

Oxidation of dehydro-ustic acid by alkaline H_2O_2

Dehydro-ustic acid (0·2582 g.) was dissolved in cold N-NaOH (10·0 ml.), and H_2O_2 ('100-vol.', 5 ml.) was added. After standing 35 min. when the yellow colour had faded and a precipitate was no longer given with Brady's reagent, the solution was acidified with N-H_2SO_4 (11·0 ml.), and steam-distilled exhaustively on the water pump at 40–45° for the estimation of volatile acid. The distillate required 9·9 ml. of 0·1N-NaOH for neutralization corresponding to 104% of the theoretical for 1 mol. volatile acid/mol. of dehydro-ustic acid. The titrated distillate was evaporated to dryness and converted into the *p*-bromophenacyl derivative in the usual way. The crude *p*-bromophenacyl derivative was purified by repeated recrystallization from light petroleum, b.p. 60–80°, and finally by sublimation in a high vacuum at 80–90°. It was obtained as colourless crystals, m.p. 87–89° not depressed on admixture with authentic *p*-bromophenacyl acetate, m.p. 86·5–88·5°, prepared from sodium acetate. (Found: C, 46·6; H, 3·4; Br, 30·9. Calc. for $C_{10}H_9O_3Br$: C, 46·7; H, 3·5; Br, 31·1%.) The volatile acid obtained in the oxidation thus consisted essentially of acetic acid together with small amounts of some other volatile acid.

The acidified steam-distillation residue was extracted three times with an equal volume of ethyl acetate, and the extracts were evaporated to give a dry solid (m.p. 188–190°, decomp., wt. 0·18 g., 83% yield). This was crystallized twice from ethyl acetate-benzene, once with charcoal, then from acetone-benzene giving colourless, flat, hexagonal prisms and needles, m.p. 192–193° with decomposition to a yellow liquid which partially resets on cooling, remelts at 170–176° with the formation of a sublimate, probably of the anhydride, of nearly colourless crystalline rods, m.p. 174–178°. (Found: C, 47·6; H, 3·4; OCH_3, 13·6, 13·5; *C*-CH_3, 1·2. $C_9H_8O_7$ requires: C, 47·4; H, 3·5; $1OCH_3$, 13·6%; *C*-CH_3, nil.) This acid, *4:6-dihydroxy-3-methoxyphthalic acid*, gives with aqueous $FeCl_3$ an intense purple colour which is indistinguishable from that given by ustic acid and dehydro-ustic acid.

In a larger-scale preparation using dehydro-ustic acid (2·0 g.) dissolved in water (250 ml.) and with the addition of H_2O_2 ('100-vol.', 50 ml.) and 2N-NaOH (50 ml.), 0·95 g. of pure 4:6-dihydroxy-3-methoxyphthalic acid, m.p. 192° (decomp.) was obtained.

Methylation of the acid $C_9H_8O_7$. *Formation of* 3:4:6-*trimethoxyphthalic acid*

A mixture of the above acid, $C_9H_8O_7$ (0·2 g.), dimethyl sulphate (1·0 ml.), anhydrous K_2CO_3 (1·0 g.) and dry acetone (20 ml.) was refluxed for 1·5 hr. Volatile solvents were removed on the water pump and the residue was refluxed for 20 min. with N-NaOH (20 ml.). The clear colourless solution was cooled, acidified with 2N-HCl and extracted with ethyl

acetate. Removal of the solvents from the extract left a solid (0·20 g.) which was crystallized from water (4 ml.) (charcoal). 3:4:6-trimethoxyphthalic acid (0·18 g.) separated, m.p. 209–211° not depressed on admixture with synthetic material. (Found, on air-dried material: C, 48·1; H, 5·0; *C*-CH_3, 2·7. Calc. for $C_{11}H_{12}O_7 . H_2O$: C, 48·2; H, 5·2%; *C*-CH_3, nil.) It was also converted into the anhydride (see p. 63), m.p. 210–211°, unchanged on mixing with the synthetic anhydride. (Found: C, 55·5; H, 3·9. Calc. for $C_{11}H_{10}O_6$: C, 55·5; H, 4·2%.)

Decarboxylation of dehydro-ustic acid

(*a*) *In hot dilute acid.* A solution of hydrated dehydro-ustic acid (0·5470 g.) in 2 N-H_2SO_4 (20 ml.) was boiled under reflux and a slow stream of CO_2-free N_2 was passed through the solution. The issuing gas was scrubbed, first through Brady's reagent and then through 0·2 N-aqueous $Ba(OH)_2$. After 6·7 hr. the Brady's reagent was still clear and 48·4% of 1 mol. equiv. CO_2 was absorbed in the $Ba(OH)_2$. The olive-green reaction solution was diluted with water to 40 ml. and extracted three times with an equal volume of ethyl acetate. The combined extracts were separated into acidic and phenolic fractions by extraction with fresh aqueous 1% $NaHCO_3$. The acidic fraction (0·30 g.) consisted of unchanged dehydro-ustic acid. The phenolic fraction, a viscous olive-brown oil (0·22 g.), was hardened with petrol to a greyish-white powder (0·19 g.) which was crystallized from benzene (15 ml.) (charcoal) to yield yellow prisms, m.p. 98–100°. Further recrystallization from benzene raised the m.p. to 100–101° without decomposition. (Found: C, 57·3, 57·1; H, 4·9; 4·8; OCH_3, 14·8. $C_{10}H_{10}O_5$ requires: C, 57·1; H, 4·8; 1OCH_3, 14·8%.) Decarboxydehydro-ustic acid (3:5-*dihydroxy-2-methoxyphenyl methyl diketone*) is very soluble in water. Its aqueous solution (pH ~ 4·5) is yellow and turns orange when made alkaline. It gives an immediate yellow precipitate with Brady's reagent. With aqueous $FeCl_3$ a dirty blue colour is observed changing rapidly to brown and then to green, the green colour slowly deepening on standing. No $FeCl_3$ colour is observed in ethanol.

(*b*) *In quinoline-copper chromite.* A mixture of hydrated dehydro-ustic acid (0·4916 g.) in purified quinoline (15 ml.) and copper chromite (0·25 g.) was heated, in an atmosphere of N_2 as in method (*a*), at 120–150° for 70 min. CO_2 equivalent to 102% theoretical for 1 mol. CO_2 was evolved. The reaction mixture was acidified and extracted with ethyl acetate. The extract, which was very dark in colour, was purified as described in method (*a*). The crude crystalline phenolic fraction (0·26 g.), m.p. 96–99°, was recrystallized from benzene (charcoal) yielding decarboxydehydro-ustic acid (0·145 g), m.p. 98–99·5°, unchanged on admixture with material prepared by method (*a*).

The *quinoxaline derivative* was prepared by boiling a solution of the diketone (0·10 g.) and *o*-phenylenediamine hydrochloride (0·10 g.) in ethanol (10 ml.). After 30 min. the solution was poured into water to give a clear yellow solution. It was made alkaline and extracted three times with ether to remove unchanged amine, then acidified to congo red and extracted again three times with an equal volume of ether. The yellow solid (0·10 g.) remaining after removal of the solvent melted at 264°. After crystallizing twice from a mixture of ethanol (7 ml.) and water (40 ml.), once with charcoal, the quinoxaline derivative separated in yellow rhombic prisms, m.p. 264–266°, with darkening and sublimation. (Found: C, 68·2, 68·1; H, 5·0, 4·9; N, 9·6; OCH_3, 11·7, 11·6. $C_{16}H_{14}O_3N_2$ requires: C, 68·1; H, 5·0; N, 9·9; 1OCH_3, 11·0%.) The compound does not fluoresce in acetone or benzene solution. In conc. H_2SO_4 it gives a brown solution unchanged on warming.

Oxidation of decarboxydehydro-ustic acid with alkaline H_2O_2

The method described for the oxidation of dehydro-ustic acid, using excess H_2O_2 (see p. 63) gave only a low yield and was therefore modified. Decarboxydehydro-ustic acid (0·258 g.) was dissolved in water (50 ml.), and N-NaOH (10·0 ml.) was added yielding an orange-red solution. H_2O_2 ('100 vol.') was added 6 drops at a time, with shaking, at intervals of 3 min., testing with Brady's reagent before each addition. After addition of 0·4 ml. H_2O_2 Brady's reagent gave only a slight precipitate. The brown solution was now treated with N-H_2SO_4 (11·0 ml.), diluted to about 100 ml. and distilled exhaustively on the water pump at 35–40° for the estimation of the volatile acid. The distillate required 11·66 ml. 0·1 N-NaOH for neutralization, corresponding to 95% of the theoretical for 1 mol. volatile acid/mol. of decarboxydehydro-ustic acid. The titrated distillate was converted in good yield into the *p*-bromophenacyl derivative which was purified as described previously, m.p. 85–86° not depressed on admixture with synthetic *p*-bromophenacyl acetate. No other *p*-bromophenacyl derivative was detected.

The distillation residue, which had become browner during the exhaustive evaporation, was extracted three times with ethyl acetate (50 ml.) and the extracts were evaporated to give a dark gummy solid (0·20 g.). By crystallization from ethyl acetate-benzene-petrol and by sublimation of the residues, a yield of 0·12 g. (53% of the theoretical) of material melting from 197° was obtained.

A purer product resulted from a repeat experiment in which the distillation was omitted. The crude product (0·37 g. from 0·50 g. decarboxydehydro-ustic acid) was crystallized from acetone (4 ml.) and benzene (40 ml.). Colourless crystals (weight 0·26 g., m.p. 205–207° without decomposition) were obtained and were prepared for analysis by sublimation in a high vacuum at 120–130°; m.p. 205–207°. (Found: C, 52·3; H, 4·4; OCH_3, 16·7. $C_9H_8O_5$ requires: C, 52·2; H, 4·4; 1OCH_3, 16·9%.) The oxidation product, 3:5-*dihydroxy-2-methoxybenzoic acid*, gives with aqueous $FeCl_3$ first a greyish-brown colour quickly deepening to a true brown and becoming deeper and redder on standing. In ethanol solution $FeCl_3$ gives no colour reaction.

The oxidation acid (0·17 g.) was methylated by the dimethyl sulphate-K_2CO_3-acetone method described for the methylation of the acid $C_9H_8O_7$ (see p. 63). The crude methylation product (0·15 g.) was crystallized from water (15 ml.) with charcoal. 2:3:5-Trimethoxybenzoic acid was thus obtained as colourless needles, m.p. 102–103°, unchanged after sublimation in a high vacuum at 90–95°. The melting point was not depressed on admixture with authentic synthetic 2:3:5-trimethoxybenzoic acid, m.p. 101–103° (see p. 65). (Found: C, 56·7; H, 6·1; OCH_3, 43·7. Calc. for $C_{10}H_{12}O_5$: C, 56·6; H, 5·7; 3OCH_3, 43·7%.)

The oxidation acid (0·10 g.) was also ethylated by the same method using diethyl sulphate in place of dimethyl sulphate. On acidification of the alkaline hydrolysis solution the ethylation product separated as an oil which slowly crystallized (weight 0·045 g., m.p. 79–81°). It was sublimed in a high vacuum at 80–90°. The colourless sublimate of 3:5-*diethoxy-2-methoxybenzoic acid* melted at 81–83° not de-

pressed on admixture with an authentic synthetic specimen, m.p. 82–83° (see below). The mixture reset on cooling and remelted at the same temperature. (Found: C, 60·0; H, 6·7; alkoxy calc. as OCH_3, 38·3. $C_{12}H_{16}O_5$ requires: C, 60·0; H, 6·7; 3 alkoxy groups calc. as OCH_3, 38·7%.)

SYNTHESES

(*a*) 2:3:5-*Trihydroxybenzoic acid.* The method used followed the general directions given by Baker & Brown (1948). 3:5-Dihydroxybenzoic acid (0·77 g.) (*Organic Syntheses*, **21**, 27) was dissolved in 2N-NaOH (12·5 ml.), and $K_2S_2O_8$ solution (1·35 g. in 35 ml. water) was added during 3·5 hr. with stirring at 10–15°. After stirring for a further 0·5 hr. the deep-red solution was held at room temperature overnight. It was then acidified with HCl to congo red and was extracted four times with half its volume of ether. This extract, on evaporation, left a residue of impure starting material. The extracted aqueous layer was treated with conc. HCl (5 ml.), hydrolysed for 30 min. on the steam bath, cooled and extracted three times with an equal volume of ether. On removal of the solvent there remained a brown gummy solid (0·30 g.) which was dissolved in ethyl acetate (10 ml.), and benzene (60 ml.) was added. A dark flocculent precipitate separated and was removed by filtration. The filtrate, on evaporation, left a dry brown solid (0·20 g.) which was crystallized from water (3 ml.), but the product (0·13 g.) contained some green impurity which was removed by sublimation. The buff-coloured sublimate (0·06 g.) was crystallized twice from water with charcoal and then once more from water. 2:3:5-Trihydroxybenzoic acid separated as pale-yellow silky needles (0·03 g.), m.p. 226–227° with decomposition and incompletely, darkening above this temperature and melting completely at 263–280°. (Found, on air-dried material: C, 40·8; H, 5·0; loss at 100°/16 mm., 17·4. $C_7H_6O_5.2H_2O$ requires: C, 40·8; H, 4·9; loss of $2H_2O$, 17·5%. On material dried at 100°/16 mm.: C, 49·2; H, 3·8. $C_7H_6O_5$ requires: C, 49·4; H, 3·6%.) Aqueous solutions of 2:3:5-trihydroxybenzoic acid are not very stable and when exposed to air at room temperature turn brown after some hours. With aqueous $FeCl_3$ they give a transient green colour changing rapidly to brown. In ethanol a more stable bluish green colour is observed, but this also fades to brown after 1 hr. Since this work was completed the synthesis of 2:3:5-trihydroxybenzoic acid has been described by Corbett, Hassall, Johnson & Todd (1950), who record m.p. 223° (decomp.).

(*b*) 2:3:5-*Trimethoxybenzoic acid.* Hydrated 2:3:5-trihydroxybenzoic acid (0·10 g.), prepared as above, was methylated by the dimethyl sulphate-K_2CO_3-acetone method. The crude product (0·09 g.), m.p. 97–100°, was crystallized from water (10 ml.), m.p. 101–102° without decomposition, wt. 0·065 g. Sublimation in a high vacuum at 90–95° raised the m.p. to 101–103°. Smith & Laforge (1931) record the m.p. as 105°; Baker, Brown & Scott (1939) give 104°. (Found: C, 56·1; H, 5·5; OCH_3, 43·7. Calc. for $C_{10}H_{12}O_5$: C, 56·6; H, 5·7; $3OCH_3$, 43·7%.)

(*c*) 3:5-*Diethoxy-2-hydroxybenzoic acid.* 3:5-Dihydroxybenzoic acid (0·77 g.) was oxidized with $K_2S_2O_8$, and the unchanged starting material was removed as described in section (*a*) above. The solution was then treated gradually with diethyl sulphate (5 ml.) and 2N-NaOH with stirring at 45–55° during 2 hr. The mixture was acidified and extracted with ether, the extract being rejected. The aqueous layer was hydrolysed with conc. HCl (5 ml.) for 30 min. on the water bath when some tarry matter separated. The whole was extracted with ether (3 × 50 ml.) and the ether was re-extracted with 0·5N-$NaHCO_3$ and then with water. The $NaHCO_3$ extract and wash water were acidified, re-extracted with ether, and the ether removed leaving a partly crystalline residue (0·20 g.). This residue was extracted with boiling benzene, the benzene was removed by evaporation and the resulting solid (0·155 g.) was crystallized from water with charcoal. 3:5-*Diethoxy-2-hydroxybenzoic acid* separated as nearly colourless plates, m.p. 125–127° (0·05 g.). (Found: C, 58·3; H, 6·4; OC_2H_5, 39·5. $C_{11}H_{14}O_5$ requires: C, 58·4; H, 6·2; $2OC_2H_5$, 39·8%.) The acid gives a blue colour with $FeCl_3$ in aqueous solution and a greenish blue in ethanol.

(*d*) 3:5-*Diethoxy-2-methoxybenzoic acid.* The above 3:5-diethoxy-2-hydroxybenzoic acid (0·04 g.) was methylated by the dimethyl sulphate-K_2CO_3-acetone method. The crude product (0·035 g.), m.p. 78–80°, was sublimed at 80–90° in a high vacuum, recrystallized from light petroleum b.p. 40–60° (3 ml.), then resublimed. 3:5-*Diethoxy-2-methoxybenzoic acid* sublimed as a colourless solid, m.p. 82–83°. (Found: C, 60·1; H, 6·7. $C_{12}H_{16}O_5$ requires: C, 60·0; H, 6·7%.)

Methylation of a degradation product of citromycetin

The methyl ester of *O*-dimethylcitromycetin was hydrolysed with ethanolic KOH and the resulting dimethoxyhydroxyphthalic acid was isolated as described by Hetherington & Raistrick (1931). It melted at 180° with loss of water, reset on cooling, and remelted at 197–201°. Its aqueous solution gave a pure purple colour with $FeCl_3$. This compound (0·15 g.) was methylated by the dimethyl sulphate-K_2CO_3-acetone method described on p. 63. After acidification colourless rhombic prisms, (0·07 g.) separated, m.p. 210·5–212°, after softening at 180° with loss of water. When mixed with 3:4:6-trimethoxyphthalic acid, m.p. 211–212°, obtained by degradation of ustic acid (see p. 63), it melted at 211·5–212·5°, resetting on cooling and remelting at the same temperature.

The above 3:4:6-trimethoxyphthalic acid (0·05 g.) was converted into the anhydride by heating in an open tube in an oil bath to 220° when it melted and began to sublime. The whole melt was now sublimed *in vacuo* at 100–150°. The sublimate, very pale yellow in colour, melted at 211–212° and did not depress the melting point of synthetic 3:4:6 trimethoxyphthalic anhydride (see p. 63). The mixture reset on cooling and remelted at the same temperature. (Found: C, 55·8; H, 4·3. $C_{11}H_{10}O_6$ requires: C, 55·5; H, 4·2%.)

SUMMARY

1. A new, colourless, crystalline mould metabolic product, *ustic acid*, $C_{11}H_{12}O_7$, m.p. 169–170° (decomp.), has been isolated from culture filtrates of four different strains of *Aspergillus ustus* (Bainier) Thom & Church which were grown on Raulin-Thom solution.

2. A number of derivatives and breakdown products of ustic acid are described.

3. Ustic acid has been shown to be 2:4-dihydroxy-5-methoxybenzoic acid with an unbranched side chain $—C_3H_5O_2$ in position 6.

4. The most favoured structure for this side chain is —CHOH.CO.CH_3, though many of the pro-

perties of ustic acid are as well illustrated by the side chains $-CO.CHOH.CH_3$, $-C(OH)=C(OH).CH_3$, $-CH(O-).C(OH).CH_3$, $-C(OH).CH(-O-).CH_3$ or by equilibrium mixtures of two or more of these five tautomers, depending on the experimental conditions obtaining when any particular test is carried out.

5. The close structural relationship existing between ustic acid and a number of other mould metabolic products and lichen acids is pointed out.

6. The synthesis of 3:5-*diethoxy*-2-*hydroxybenzoic acid* and of 3:5-*diethoxy*-2-*methoxybenzoic acid* is described.

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REFERENCES

Asahina, Y. (1939). *Fortschr. Chem. org. Naturstoffe.* **2**, 27.
Asahina, Y. & Fuzikawa, F. (1934). *Ber. dtsch. chem. Ges.* **67**, 163, 169.
Asahina, Y. & Fuzikawa, F. (1935). *Ber. dtsch. chem. Ges.* **68**, 2020.
Asahina, Y. & Hashimoto, A. (1933). *Ber. dtsch. chem. Ges.* **66**, 641.
Asahina, Y., Kanaoka, Y. & Fuzikawa, F. (1933). *Ber. dtsch. chem. Ges.* **66**, 649.
Asahina, Y. & Nogami, H. (1934). *Ber. dtsch. chem. Ges.* **67**, 805.
Asahina, Y. & Nogami, H. (1935). *Ber. dtsch. chem. Ges.* **68**, 77, 1500.
Asahina, Y. & Nonomura, S. (1935). *Ber. dtsch. chem. Ges.* **68**, 1698.
Asahina, Y. & Yasue, M. (1936). *Ber. dtsch. chem. Ges.* **69**, 643.
Asahina, Y. & Yasue, M. (1937). *Ber. dtsch. chem. Ges.* **70**, 206.
Bain, D., Perkin, W. H. & Robinson, R. (1914). *J. chem. Soc.* **105**, 2392.
Baker, W. & Brown, N. C. (1948). *J. chem. Soc.* p. 2303.
Baker, W., Brown, N. C. & Scott, J. A. (1939). *J. chem. Soc.* p. 1922.
Bargellini, G. (1914). *Gazz. chim. ital.* **44**, I, 182.
Calam, C. T., Clutterbuck, P. W., Oxford, A. E. & Raistrick, H. (1947). *Biochem. J.* **41**, 458.
Clutterbuck, P. W., Oxford, A. E., Raistrick, H. & Smith, G. (1932). *Biochem. J.* **26**, 1441.
Corbett, R. E., Hassall, C. H., Johnson, A.W. & Todd, A. R. (1950). *J. chem. Soc.* p. 1.
Doering, W. E., Dubos, R. J., Noyce, P. S. & Dreyfus, R. (1946). *J. Amer. chem. Soc.* **68**, 725.
Fuson, R. C., Byers, D. J. & Rachlin, A. I. (1942). *J. Amer. chem. Soc.* **64**, 2891.
Fuson, R. C. & Corse, J. (1939). *J. Amer. chem. Soc.* **61**, 975.
Fuson, R. C., Corse, J. & McKeever, C. H. (1939). *J. Amer. chem. Soc.* **61**, 2010.
Fuson, R. C., Corse, J. & Welldon, P. B. (1941). *J. Amer. chem. Soc.* **63**, 2645.
Fuson, R. C. & Horning, E. C. (1940). *J. Amer. chem. Soc.* **62**, 2962.
Fuson, R. C. & Kelton, S. C. (1941). *J. Amer. chem. Soc.* **63**, 1500.
Fuson, R. C., McKeever, C. H. & Behr, L. C. (1941). *J. Amer. chem. Soc.* **63**, 2648.
Fuson, R. C., McKeever, C. H. & Corse, J. (1940). *J. Amer. chem. Soc.* **62**, 600.
Fuson, R. C. & Scott, S. L. (1942). *J. Amer. chem. Soc.* **64**, 2152.
Fuson, R. C., Scott, S. L., Horning, E. C. & McKeever, C. H. (1940). *J. Amer. chem. Soc.* **62**, 2091.
Fuson, R. C., Scott, S. L. & Lindsey, R. V. (1941). *J. Amer. chem. Soc.* **63**, 1679.
Herbert, R. W., Hirst, E. L., Percival, E. G. V., Reynolds, R. J. W. & Smith, F. (1933). *J. chem. Soc.* p. 1270.
Hetherington, A. C. & Raistrick, H. (1931). *Phil. Trans.* B, **220**, 209.
Hogeboom, G. H. & Craig, L. C. (1946). *J. biol. Chem.* **162**, 363.
Kling, A. (1905). *C.R. Acad. Sci., Paris*, **140**, 1456.
Kurung, J. M. (1945). *Science*, **102**, 11.
Oxford, A. E. & Raistrick, H. (1933). *Biochem. J.* **27**, 634.
Raistrick, H. & Smith, G. (1936). *Biochem. J.* **30**, 1315.
Raper, K. B. & Thom, C. (1949). *Manual of the Penicillia*, p. 172. London: Baillière, Tindall and Cox.
Smith, L. E. & Laforge, F. B. (1931). *J. Amer. chem. Soc.* **53**, 3072.
Stickings, C. E. (1949). *Biochem. J.* **44**, xxiii.
Thom, C. & Raper, K. B. (1945). *Manual of the Aspergilli*, p. 178. London: Baillière, Tindall and Cox.

Studies in the Biochemistry of Micro-organisms

83. FUSCIN, A METABOLIC PRODUCT OF *OIDIODENDRON FUSCUM* ROBAK. PART 2. DERIVATIVES AND DEGRADATION PRODUCTS

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Michael (1948) described the preparation, properties and antibacterial activity of fuscin, $C_{15}H_{16}O_5$, a mould metabolite which he isolated from culture filtrates of laboratory cultures of *Oidiodendron fuscum* Robak. A number of derivatives and degradation products of fuscin have been prepared and are described in the present communication. It is hoped to discuss their bearing on the molecular constitution of fuscin in a later paper.

EXPERIMENTAL

Derivatives of fuscin

Mono-sodium salt of fuscin. Fuscin (0·1 g.) was dissolved in saturated aqueous $NaHCO_3$ (50 ml.) and kept at room temperature for 1 week. The purple solution slowly deposited dark-purple cubes of the *monosodium salt* which were separated by filtration, washed quickly with a minimum of water and dried. (Found: Na, 6·3. $C_{15}H_{15}O_5Na\ 4H_2O$ requires Na, 6·2%.) It is much more soluble in ethanol than in water.

Addition compounds of fuscin

(*a*) *With hydrochloric acid.* Dry gaseous HCl was passed through a suspension, cooled in ice and salt, of fuscin (0·2 g.) in anhydrous ether (20 ml.). The fuscin slowly dissolved (0·5 hr.) to a pale cream-coloured solution from which colourless rods separated (1 hr.). They were collected, washed with anhydrous ether and dried *in vacuo* over KOH. The *compound*, 0·14 g., melted at 210° (decomp.) after darkening at 130–150°. (Found: C, 57·1; H, 5·4; Cl, 12·1. $C_{15}H_{16}O_5 \cdot HCl$ requires: C, 57·6; H, 5·5; Cl, 11·3%.) It is stable in contact with anhydrous ether or conc. HCl, but decomposes into fuscin and HCl in contact with ethanol or water. When 0·0352 g. in water (5 ml.) was heated at 100° for 1 hr. the solution became pale yellow and typical orange diamond-shaped crystals of fuscin separated. These were collected and the cold filtrate and washings were titrated with 0·1 N-NaOH to phenolphthalein. (Found: 1·15 ml. 0·1 N-NaOH. $C_{15}H_{16}O_5 \cdot HCl \rightarrow HCl$ requires 1·13 ml.) Dihydrofuscin, similarly treated with HCl in ether, did not give a hydrochloride.

(*b*) *With methanol. Fuscin 'methanolate'.* Methanolic KOH (15 ml. of a 25% (w/v) solution) was added to a suspension of fuscin (1 g.) in methanol (5 ml.). The resulting brownish solution was chilled, diluted with iced water (80 ml.) and rapidly acidified with ice-cold 5 N-HCl (12–15 ml.). The buff-coloured precipitate (0·82 g.) which formed was purified by crystallization from ether or, with more loss, from ethanol. *Fuscin 'methanolate'* was thus obtained as almost colourless short thick tablets. (Found: C, 62·4, 62·2; H, 6·6, 6·8; CH_3O, 11·0; active hydrogen, in pyridine 0·35, in anisole 0·23. $C_{16}H_{20}O_6$, i.e. $C_{15}H_{16}O_5 \cdot CH_3OH$, requires: C, 62·3; H, 6·5; $1CH_3O$, 10·1; 1 active H, 0·33%.) It forms a pure red solution in dil. NaOH, but is insoluble in aqueous $NaHCO_3$ (cf. fuscin). The substance has no sharp melting point. On heating, the colour changes to a pure orange at 160° and it then melts with decomposition at 210–225°, depending on the rate of heating. This is due to reversion to fuscin as was shown by the recovery of fuscin from the orange residue resulting from holding the substance at 200° for a short time. The substance gives a pure blue colour in ethanol with a trace of ethanolic $FeCl_3$, becoming green with a slight excess, and red and finally almost colourless on dilution with water. Fuscin itself gives a purplish red colour in ethanol. The substance appears to be a substituted quinol, since on shaking its colourless $CHCl_3$ solution with aqueous, slightly acid, $FeCl_3$ the $CHCl_3$ layer became intensely purple, and gave a dark-purple solid on removal of the solvent. The solid gave an intense orange-red colour with conc. H_2SO_4, and was reduced in $CHCl_3$ solution with aqueous $Na_2S_2O_4$ with reformation of the parent 'methanolate'.

Diacetyl derivative. Acetylation of the 'methanolate' with acetic anhydride and anhydrous sodium acetate in the usual way gave a good yield of a *diacetate* crystallizing from ethanol in colourless needles, m.p. 158–159°. (Found: C, 61·1; H, 6·3; CH_3O. 7·4. $C_{20}H_{24}O_8$ requires: C, 61·2; H, 6·2; $1CH_3O$, 7·9%.)

(*c*) *With thiourea. Fuscin thiuronium chloride.* Fuscin (0·2 g.) was added to a solution of thiourea (0·3 g.) in 2 N-HCl (1·5 ml.). The initial orange colour disappeared after shaking for 0·5 hr. and a fine white precipitate was formed. This was collected after chilling for 3 hr., washed well with 2 N-HCl and dried. The compound, *fuscin thiuronium chloride*, forms fine white needles, wt. 0·206 g., m.p. 205–208°. (Found: C, 46·1; H, 5·5; N, 6·95; Cl, 8·75; S, 7·9. $C_{16}H_{23}O_6N_2SCl$, i.e. $C_{15}H_{16}O_5 + CS(NH_2)_2 + HCl + H_2O$ requires: C, 47·2; H, 5·7; N, 6·9; Cl, 8·7; S, 7·9%.) In contact with water it decomposes rapidly into its constituents. Its solution in NaOH rapidly develops the purple colour of the Na salt of fuscin. Its solution in ethanol, which is quite stable, gives a reddish brown colour with ethanolic $FeCl_3$.

Acetylation of fuscin

(*a*) *With acetic anhydride and conc.* H_2SO_4. A suspension of fuscin (0·5 g.) in 6 ml. of a mixture of acetic anhydride (10 ml.) and conc. H_2SO_4 (0·2 ml.) was held at 50° for 2 min. The resulting pale-yellow solution, on treatment with ice and water, yielded a colourless solid (0·68 g.) which, on re-

crystallization from ethanol (15 ml.), gave the pure *acetyl derivative* as characteristic colourless prisms, 0·5 g., m.p. 193–194°. (Found: C, 59·8, 59·8; H, 5·8, 5·7; $CH_3.CO$, 30·4. $C_{21}H_{24}O_9$, i.e. the triacetate of $C_{15}H_{16}O_5.H_2O$, requires: C, 60·0; H, 5·75; $3CH_3.CO$, 30·7%.)

(*b*) *With acetic anhydride and anhydrous sodium acetate.* A mixture of acetic anhydride (1 ml.) and anhydrous sodium acetate (0·2 g.) was heated at 145–150° until complete solution was effected. After cooling to 100°, fuscin (0·1 g.) was added and the mixture was held at 100° for 2 min. The resulting yellow solution, on pouring into ice and water, gave 0·11 g. of a cream-coloured solid. On crystallization from glacial acetic acid (1 ml.) and water (2 ml.) characteristic colourless prisms of the acetyl derivative were obtained, wt. 66 mg., m.p. 193° alone or in admixture with the acetyl derivative of fuscin prepared by acetylation with acetic anhydride and conc. H_2SO_4 (see section *a* above).

Derivatives of dihydrofuscin

Dihydrofuscin, prepared from fuscin by reduction with $Na_2S_2O_4$ or catalytically with H_2 and Pd, has been described by Michael (1948).

(*a*) *Diacetyldihydrofuscin.* A warm solution of fuscin (0·2 g.) in glacial acetic acid (3 ml.) was reduced with Zn dust (0·2 g.). To the mixture, initially orange, but quickly becoming colourless, acetic anhydride (3 ml.) and anhydrous sodium acetate (0·5 g.) were added and the whole was boiled under reflux for 0·5 hr. The colourless product, wt. 0·33 g., m.p. 166–168°, formed on pouring the acetylation mixture into ice and water, was recrystallized, wt. 0·18 g., from ethanol (5 ml.). *Diacetyldihydrofuscin* was thus obtained as colourless prisms, m.p. 168–169°. (Found: C, 62·4; H, 6·2; $CH_3.CO$, 23·0. $C_{19}H_{22}O_7$ requires: C, 63·0; H, 6·1; $2CH_3.CO$, 23·75%.) It is insoluble in $NaHCO_3$. It is also formed by the direct acetylation of dihydrofuscin.

(*b*) *Dimethyl derivatives: melting points* 98 *and* 252°. A solution of fuscin (2 g.) in $CHCl_3$ (100 ml.) was reduced by shaking with a solution of $Na_2S_2O_4$ (10 g.) in water (40 ml.). The colourless $CHCl_3$ solution was evaporated to dryness in a stream of N_2. The residual dihydrofuscin was methylated at room temperature and under N_2 with dimethyl sulphate (20 ml.) and 2N-NaOH (125 ml.), added in portions. The residual alkaline liquid was acidified with HCl and extracted with ether. On removal of the solvent a partially crystalline residue remained. This was washed with a little dry ether and the residual crystals, 0·05 g., m.p. 238–242°, were recrystallized from ethanol. A *dimethyl* derivative, m.p. 252°, was thus obtained in colourless square plates. (Found: C, 66·1; H, 6·9; CH_3O, 20·5. $C_{17}H_{22}O_5$ requires: C, 66·6; H, 7·2; $2CH_3O$, 20·25%.)

The ethereal mother liquors from the crystals, m.p. 238–242°, were evaporated to a syrup which slowly crystallized on standing and was then drained and washed with ether. The solid, 0·4 g., m.p. 93–96°, on recrystallization from ether-light petroleum gave a second *dimethyl* derivative, m.p. 98°, in large colourless plates. (Found: C, 66·95; H, 7·2; CH_3O, 21·0. $C_{17}H_{22}O_5$ requires: C, 66·6; H, 7·2; $2CH_3O$, 20·25%.)

Both the dimethyl derivatives were insoluble in 2N-NaOH either in the cold or on heating. Neither of them gave a $FeCl_3$ colour reaction in ethanol or any precipitate with Brady's reagent.

(*c*) *Dihydrofuscin 2:4-dinitrophenylhydrazone.* Dihydrofuscin (0·05 g.) dissolved in ethanol (5 ml.) was treated with Brady's reagent (0·3% 2:4-dinitrophenylhydrazine in 2N-HCl; 50 ml.). The precipitate which formed quickly was collected (0·07 g.) and crystallized from benzene (15–20 ml.). *Dihydrofuscin 2:4-dinitrophenylhydrazone* forms yellow rhombic plates, m.p. 274°, when heated quickly. (Found, on sample dried in a high vacuum at 150°: C, 55·0; H, 5·2; N, 12·3. $C_{21}H_{22}O_8N_4$ requires: C, 55·0; H, 4·8; N, 12·2%.) Fuscin, when treated similarly, does not form a dinitrophenylhydrazone.

Action of diazomethane on fuscin. Formation of methylhomofuscin

A solution of fuscin (2·5 g.) in $CHCl_3$ (180 ml.) was treated with an excess of ethereal diazomethane. The reaction proceeded smoothly and quickly. Removal of the solvent left a gum which was crystallized from dry ether giving *methylhomofuscin* (2·49 g.) as colourless prisms, m.p. 153–154°, raised to a constant m.p. 155° by sublimation in a high vacuum at 140–150° and subsequent crystallization from ether. (Found: C, 67·0, 67·0; H, 6·7, 6·7; N, nil; CH_3O, 11·5, 11·6. $C_{17}H_{20}O_5$, i.e. $C_{15}H_{15}O_4 + CH_2 + OCH_3$, requires: C, 67·1; H, 6·6; $1CH_3O$, 10·2%.) The substance is readily soluble in $CHCl_3$, ethanol or acetone, less so in ether or cold water (about 0·5%). It dissolves readily in 0·01N-NaOH in the cold to a colourless solution, but is insoluble in aqueous $NaHCO_3$. It gives no colour with ethanolic $FeCl_3$, but an intense blue colour with cold 50% (v/v) HNO_3. Like fuscin, but unlike dihydrofuscin, it does not form a 2:4-dinitrophenylhydrazone. It does not appear to react with hydrazine hydrate in ethanol, and hence it is probably not a methyl ester. Unlike fuscin and dihydrofuscin (see p. 71) it does not give acetaldehyde on alkaline hydrolysis.

Acetylation product. Methylhomofuscin (0·1 g.) was treated in the usual way with acetic anhydride (2 ml.) and sodium acetate (0·2 g.). Since, on dilution with water, the acetylation mixture gave no precipitate, it was extracted with ether. The residue (0·07 g.) on removal of the ether consisted of methylhomofuscin hydrate, m.p. 180° (see next section).

Methylhomofuscin hydrate. Methylhomofuscin titrates fairly sharply in the cold to phenolphthalein as a monobasic acid. (Titration equivalent. Found: 289. $C_{17}H_{20}O_5$ requires 304.) A solution of methylhomofuscin (0·15 g.) in 0·1N-NaOH (5 ml.) was held for 1 hr. at room temperature and then neutralized by the addition of 0·1N-H_2SO_4 (5 ml.). A trace of white precipitate was filtered off. The filtrate was acidified with 0·1N-H_2SO_4 (5 ml.) and extracted with ether. On removal of the solvent the solid residue (0·11 g.) was crystallized from dry ether, and *methylhomofuscin hydrate* was obtained as large colourless bipyramidal crystals, m.p. 180°. (Found: C, 62·9; H, 6·9; CH_3O, 9·7. $C_{17}H_{22}O_6$ requires: C, 63·3; H, 6·9; $1CH_3O$, 9·6%.) It is readily soluble in N-NaOH but insoluble in $NaHCO_3$. It gives no colour with $FeCl_3$ either in ethanol or in water.

Acetylation product. Acetylation of methylhomofuscin hydrate with acetic anhydride and anhydrous sodium acetate in the usual manner gave, on dilution with water, a precipitate, which on crystallization from 60% aqueous ethanol yielded the *acetate* in colourless prisms, m.p. 162°. (Found: C, 65·55; H, 6·5; CH_3O, 9·3. $C_{19}H_{22}O_6$, i.e. $C_{17}H_{22}O_6 + C_2H_2O - H_2O$, requires: C, 65·9; H, 6·4; $1CH_3O$, 9·0%.) The substance is neutral in reaction and is insoluble in $NaHCO_3$ or N-NaOH. It is a *monoacetate* and is identical with the monoacetate of *iso*methylhomofuscin (see p. 69).

Dihydromethylhomofuscin. A solution of methylhomofuscin (0·5 g.) in ethanol (50 ml.) was reduced by H_2 and a Pd-norite catalyst prepared from $PdCl_2$ (0·2 g.) and norite (0·5 g.). In 1 hr. 38·4 ml. H_2 were absorbed; absorption of 1 mol. H_2 requires 36·8 ml. The catalyst was removed by filtration and the ethanol by evaporation. The crystalline residue, on recrystallization from ethanol, gave *dihydromethylhomofuscin* (0·44 g.), m.p. 182°, as colourless short rods. (Found: C, 67·0; H, 7·4; CH_3O, 10·4; active H, in pyridine, 0·42, in anisole, 0·19. $C_{17}H_{22}O_5$ requires: C, 63·65; H, 7·2; $1CH_3O$, 10·1; 1 active H, 0·33 %.) The substance gives no colour in ethanol with ethanolic $FeCl_3$, but in aqueous ethanol it gives a brownish-red colour with an excess of aqueous $FeCl_3$. It is insoluble in $NaHCO_3$, but dissolves in N-NaOH to a colourless solution from which it is precipitated by CO_2.

Dihydromethylhomofuscin monomethyl ether. Dihydromethylhomofuscin (0·2 g.) was treated with a large excess of ethereal diazomethane for several days. Removal of the solvent left an oil which slowly crystallized. Recrystallization from aqueous ethanol gave *dihydromethylhomofuscin monomethyl ether* as colourless fine plates, m.p. 107–108°. (Found: C, 67·6, 67·8; H, 7·6; 7·4, CH_3O, 19·5. $C_{18}H_{24}O_5$ requires: C, 67·5; H, 7·55; $2CH_3O$, 19·4 %.) The substance gives no $FeCl_3$ colour. It is soluble in dil. NaOH and is precipitated from this solution by CO_2.

Compound, $C_{17}H_{21}O_5Cl$, of methylhomofuscin with HCl. Methylhomofuscin has the remarkable property of forming a stable *compound* with HCl even in aqueous solution. On the addition of 2 N-HCl to a clear, saturated aqueous solution of methylhomofuscin (0·05 g. in 10 ml. water) an immediate white precipitate of the compound is formed. It is also formed by acidification with HCl of a solution of methylhomofuscin in the minimum excess of 0·1 N-NaOH. It is most conveniently prepared as follows: conc. HCl (0·1 ml.) was added to a clear cold solution of methylhomofuscin (0·15 g.) in ethanol (2 ml.). The compound (0·19 g.) quickly crystallized in colourless broad needles which, on recrystallization from 50 % (v/v) aqueous ethanol, melted at 191° with vigorous evolution of HCl. The melt then reset and remelted at 224° owing to the formation of *iso*methylhomofuscin (see below). (Found: C, 60·2; H, 6·3; Cl, 10·3; CH_3O, 8·7. $C_{17}H_{21}O_5Cl$ requires: C, 59·9; H, 6·2; Cl, 10·4; $1CH_3O$, 9·1 %.) An aqueous ethanol solution of the compound, acidified with dil. HNO_3, gives no precipitate with $AgNO_3$. The chlorine in the molecule is, however, converted into the ionized form by boiling the substance with aqueous N-NaOH.

iso*Methylhomofuscin*

This compound was prepared by three different methods, of which the first is the most convenient.

(*a*) *By heating the compound, $C_{17}H_{21}O_5Cl$ (see above).* The compound, $C_{17}H_{21}O_5Cl$ (0·33 g.) was heated in an oil bath to 200°. HCl was evolved. The resolidified melt (0·28 g.) was crystallized from a mixture of $CHCl_3$ (1 vol.) and ether (5 vol.) yielding *isomethylhomofuscin* (0·25 g.) as colourless glistening tablets, m.p. 226°. (Found: C, 66·9; H, 6·8; CH_3O, 10·0. $C_{17}H_{20}O_5$ requires: C, 67·1; H, 6·6; $1CH_3O$, 10·2 %.) It is soluble in N-NaOH but insoluble in $NaHCO_3$. It gives no colour with $FeCl_3$ either in ethanol or in water. Unlike methylhomofuscin, *iso*methylhomofuscin gives no marked colour with 50 % aqueous HNO_3.

(*b*) *By the action of iodine on methylhomofuscin.* 0·1 N-I_2 (50 ml.) was added to a solution of methylhomofuscin (0·30 g.) in ethanol (50 ml.). After 3 hr. the residual I_2 was titrated with 0·1 N-$Na_2S_2O_3$. Only 2·87 ml. 0·1 N-I_2 was absorbed. The colourless titrated solution was diluted with much water and the resulting precipitate (0·28 g.), m.p. 180–190°, was purified by crystallization, first from aqueous ethanol, and then from ether. The resulting *iso*methylhomofuscin melted at 226° alone or in admixture with material prepared by method (*a*).

(*c*) *By boiling methylhomofuscin with* N-NaOH. In an attempt to degrade methylhomofuscin by alkaline hydrolysis, a solution of 1·0 g. of it in N-NaOH (100 ml.) was boiled for 5 hr. in a stream of N_2. The issuing gases were scrubbed through Brady's reagent. Only a trace of precipitate was obtained. The cooled hydrolysate, on acidification and heating to 90°, gave only a trace of CO_2 and an amorphous precipitate (0·37 g.) from which, by fractionation first from ether-light petroleum and then ether, 0·03 g. of *iso*methylhomofuscin, m.p. 224–226°, was isolated. The solid material (0·66 g.), obtained by ether extraction of the filtrate from the amorphous precipitate (0·37 g.) referred to above, gave, on fractionation, only amorphous products which could not be purified.

*Acetylation product. iso*Methylhomofuscin (0·1 g.) was acetylated in the usual way with acetic anhydride (1 ml.) and anhydrous sodium acetate (0·2 g.). The resulting acetyl derivative, 0·10 g. of colourless prisms, melted at 160–162° alone or in admixture with the acetyl derivative, $C_{19}H_{22}O_6$, of methylhomofuscin hydrate (see p. 68).

Degradation products of fuscin and dihydrofuscin

A. *By pyrolysis*

In this series of experiments a weighed amount of fuscin or dihydrofuscin, contained in a small distillation flask, was heated in an oil bath. The side arm of the flask was connected through a small receiving flask to bubblers, the first containing Brady's reagent, in which no precipitate appeared either with fuscin or dihydrofuscin, the others a measured amount of 0·2 N-$Ba(OH)_2$ solution. Before heating was commenced and also during heating, a stream of O_2- and CO_2-free N_2 was passed through the whole apparatus. At the end of the experiment the CO_2 absorbed in the $Ba(OH)_2$ bubblers was estimated by titration. The following results were obtained.

(1) *Fuscin:* 0·194 g. used. The substance melted at 230°, gas evolution commenced at 240–250°, and at 270–295° a colourless distillate appeared in the receiver. The heating was continued for 1 hr. at 295–320°. 0·0336 g. of CO_2, equivalent to 1·09 mol. of CO_2, was evolved. No pure solid product could be obtained from the oily distillate.

(2) *Dihydrofuscin:* 0·288 g. used. The substance melted at 204°, gas evolution commenced at 240°, and a colourless distillate collected in the side arm of the flask at 260–285°. Heating was continued for 0·5 hr. at 270–280°. 0·052 g. of CO_2, equivalent to 1·15 mol. of CO_2 was evolved. (In a repeat experiment 1·09 mol. of CO_2 was evolved.) An undistilled brown residue (0·169 g.), which could not be crystallized, remained in the flask. The distillate in the side arm solidified overnight as an almost colourless solid (wt. 0·078 g., 27 %). This was purified by extraction with hot light petroleum (100 ml.), sublimation in a high vacuum at 80–100° of the solvent-free extract and recrystallization of the sublimate from light petroleum. Colourless fern-shaped crystals, m.p. 139°, were thus obtained. (Found: C, 69·3, 69·5; H, 7·8, 7·8; *C*-CH_3, 11·8; active hydrogen, in anisole, 0·47, in pyridine,

0·34%. Mol. wt. (Rast) 202. $C_{12}H_{16}O_3$ requires: C, 69·2; H, 7·75; 2*C*-CH_3, 14·4; 1 active H, 0·49%. Mol. wt. 208.) The *substance* is insoluble in $NaHCO_3$, but forms a yellow solution in NaOH. It gives a green colour in ethanol with ethanolic $FeCl_3$, changing to reddish-purple on the addition of water. It turns pink on exposure to air and is clearly a phenol although no crystalline acetate could be obtained from it. It was also obtained by decarboxylation, either by heating above the melting point or with quinoline-copper chromite, of the acid, $C_{13}H_{16}O_5$, which was the main hydrolysis product yielded by boiling dihydrofuscin with aqueous 2N-NaOH (see section E).

B. *By heating with copper chromite and quinoline*

In these experiments the same apparatus was used as is described in section A, the operation being carried out in an atmosphere of N_2. No precipitate was obtained in the Brady's reagent with either fuscin or dihydrofuscin.

(1) *Fuscin.* Fuscin (0·9756 g.), quinoline (25 ml.) and copper chromite catalyst (Kinney & Langlois, 1931) (0·5 g.) used. CO_2 began to be evolved at 130°, and the mixture was held at 150–155° for 4·5 hr. 0·141 g. of CO_2, equivalent to 0·91 mol. of CO_2, was evolved. The cooled reaction mixture was acidified with dil. HCl and extracted with ether. On removal of the solvent there remained 0·4 g. of a dark-brown oil which could not be obtained crystalline.

(2) *Dihydrofuscin.* Dihydrofuscin (0·4039 g.), quinoline (10 ml.) and copper chromite (0·2 g.) used. CO_2 began to be evolved at 155°, and the mixture was held at 170–180° for 4 hr. 0·0600 g. of CO_2, equivalent to 0·94 mol. of CO_2, was evolved. The cooled reaction mixture was acidified with 2N-HCl (180 ml.) and extracted with six 100 ml. portions of ether. The ether residue, 0·5 g. of brown oil, was washed several times with water, dried *in vacuo*, and the brown glassy residue (0·306 g.) was extracted with hot light petroleum (2 × 50 ml.). Evaporation of the light petroleum extract to 20 ml. gave colourless crystals, m.p. 139–140°, not depressed on admixture with the substance $C_{12}H_{16}O_3$ obtained by pyrolysis of dihydrofuscin (see section A 2).

C. *By zinc dust distillation*

In these experiments fuscin and dihydrofuscin were heated to a dull red heat with Zn dust in a combustion tube in a stream of dry H_2. The issuing H_2 was passed first through a small empty receiver, then bubbled through a second receiver containing saturated ethanolic picric acid solution (3 ml.). Both receivers were cooled in ice.

(1) *Fuscin.* Fuscin (1·0 g.) and Zn dust (15 g.) were used. A yellow oil (27 mg.) collected in the cool part of the combustion tube. It smelt strongly of tar, and its ether solution showed a strong blue fluorescence. It gave no colour reaction with $FeCl_3$. When mixed with saturated ethanolic picric acid solution (2 ml.) the colour changed to dark brown. The solution was boiled for 5 min., then evaporated slowly at room temperature. Brown needles formed and were washed with water to remove excess picric acid; wt. 65 mg., m.p. 95° after softening at 82°. (Found: C, 42·3, 41·9; H, 2·3, 2·2; N, 15·15, 14·95; *C*-CH_3, 1·8. $(C_4H_4)_n.(C_6H_3O_7N_3)_n$ requires: C, 42·7; H, 2·5; N, 14·9; 1(*C*-CH_3) if $n = 3$, 1·77, if $n = 2$, 2·67%.) Hence the parent hydrocarbon is $(C_4H_4)_n$, and in view of the *C*-CH_3 estimation is probably $C_{12}H_{12}$. Its properties are different from any recorded tripicrates of known $C_{12}H_{12}$ hydrocarbons, e.g. ethylnaphthalenes or dimethylnaphthalenes, etc., and also from known picrates of hydrocarbons C_8H_8, e.g. styrene and *cyclo*-octatetraene.

(2) *Dihydrofuscin.* Dihydrofuscin (1 g.) and Zn dust (15 g.) were used. A completely liquid oil (17 mg.) collected at the cold end of the combustion tube. It gave the same picrate (orange needles, 7 mg., m.p. and mixed m.p. 95°) as was obtained from fuscin. A partially crystalline viscous oil (10 mg.), giving a blue fluorescent solution in ether, collected near the end of the Zn layer. It gave a picrate (15 mg.), m.p. 80°, in dark-brown needles. (Found: C, 50·55; H, 2·9; N, 12·4. $C_{14}H_{11}O_7N_3$, i.e. $C_8H_8.C_6H_3O_7N_3$, requires: C, 50·5; H, 3·3; N, 12·6%.)

No picrate could be isolated from the ethanolic picric acid bubbler either with fuscin or dihydrofuscin.

D. *By potash fusion of dihydrofuscin*

A nickel crucible containing dihydrofuscin (1 g.) and KOH (10·0 g.) was placed in a short wide glass tube closed with a rubber bung carrying inlet and outlet tubes, and a tap funnel. After displacing the air by O_2-free N_2 through the inlet and outlet tubes, water (4 ml.) was added to the crucible through the tap funnel. The tube and its contents were heated slowly in a metal bath to 260° till most of the water had evaporated, and were then held at 280–290° for 0·5 hr. after the melt had become quiescent. After cooling, water (10 ml.) and 13·5N-H_2SO_4 (14 ml.) were carefully added. The contents of the tube were washed into a Claisen flask with water (100 ml.) and evaporated *in vacuo* to low bulk three times, water (100 ml.) being added at the commencement of the second and third evaporations. The receiver was connected to a water trap.

Treatment of the distillate. The distillate was titrated with N-NaOH, requiring from 5 to 6 ml. in different experiments. The neutralized solution was evaporated to dryness *in vacuo*, and the dry Na salts (0·6 g.) were extracted with boiling acetone (400 ml.). The acetone-soluble and acetone-insoluble Na salts were separately converted into their *p*-bromophenacyl esters by boiling in 80% ethanol with the theoretical amount of *p*-bromophenacyl bromide, and were separately fractionated from light petroleum (b.p. 40–60°). The purified *p*-bromophenacyl ester from the acetone-soluble Na salt was obtained as colourless prisms, m.p. 68° alone or in admixture with authentic *p*-bromophenacyl *iso*valerate, m.p. 68°. (Found: C, 52·35; H, 5·1; Br, 27·9. Calc. for $C_{13}H_{15}O_3Br$: C, 52·2; H, 5·05; Br, 26·7%.) The purified *p*-bromophenacyl ester from the acetone-insoluble Na salt was obtained as long colourless glistening needles, m.p. 86° alone or in admixture with authentic *p*-bromophenacyl acetate, m.p. 86°.

Treatment of residue. The solution remaining after removal of the volatile acids was extracted with ethyl acetate. On removal of the solvent, the solid dark-brown residue (0·67 g.) was purified by crystallization from ethyl acetate-light petroleum, and was finally obtained by sublimation in a high vacuum as colourless crystals, m.p. 161°. (Found: C, 52·0, 52·4; H, 4·7, 4·6. $C_8H_8O_5$ requires: C, 52·2; H, 4·4%.) The substance, 3:4:5-*trihydroxyphenylacetic acid*, gives a steely blue colour in ethanol with ethanolic $FeCl_3$, the colour becoming a more intense blue with a tinge of purple on the addition of water.

Triacetate. 0·056 g. was acetylated with acetic anhydride (0·6 ml.) and sodium acetate (0·1 g.). 3:4:5-*Triacetoxyphenylacetic acid* was recovered by ether extraction of the acidified diluted acetylation mixture, and was purified from ether-light petroleum and finally ethanol, m.p. 196°. (Found C, 54·4; H, 5·0. $C_{14}H_{14}O_8$ requires: C, 54·2; H, 4·55%.)

Trimethyl ether. 0·2 g. was methylated for 3 days with ethereal diazomethane. The methylation residue was saponified and separated into acidic and neutral fractions. The acidic fraction (0·17 g.) was purified from ether and sublimed in a high vacuum giving colourless needles, m.p. 119°. (Found: C, 58·5; H, 6·2; CH_3O, 40·5; titration equivalent 230. Calc. for $C_{11}H_{14}O_5$: C, 58·4; H, 6·2; $3CH_3O$, 41·15%; equivalent, titrating as a monobasic acid, 226.) The substance was identified as 3:4:5-trimethoxyphenylacetic acid (Slotta & Müller, 1936), by comparison with an authentic specimen kindly supplied by Dr A. J. Ewins, F.R.S. A mixture of the methylated fusion product (m.p. 119°) with the authentic specimen (m.p. 121°) melted at 120°. The identity of the trimethyl ether was confirmed by oxidation with alkaline $KMnO_4$, when colourless needles, m.p. 167°, of 3:4:5-trimethoxybenzoic acid were obtained. The melting point was not depressed on admixture with an authentic specimen, m.p. 167°, obtained by methylation of gallic acid.

E. *By alkaline hydrolysis*

In these experiments fuscin and dihydrofuscin were hydrolysed with boiling aqueous NaOH in an atmosphere of N_2. The issuing N_2 was scrubbed during the hydrolysis directly through bubblers containing Brady's reagent. The cooled hydrolysis solution was acidified, heated to 90°, and CO_2, swept out in a current of N_2, was absorbed in baryta bubblers.

(1) *Fuscin.* Fuscin (1·0 g.) and N-NaOH (100 ml.) were used. The initial dark bluish-purple solution finally became pale yellow-brown in colour after 9·5 hr. hydrolysis. The Brady's reagent deposited a heavy bright-yellow precipitate, m.p. 148–150°, wt. 0·28 g., equivalent to 0·35 mol. of acetaldehyde 2:4-dinitrophenylhydrazone, which was identified by failure of a sample, recrystallized from light petroleum b.p. 60–80° in golden plates and yellow needles, m.p. 158–160°, to depress the m.p. of an authentic specimen, and by analysis. (Found: C, 43·1; H, 3·4; N, 24·1. Calc. for $C_9H_8O_4N_4$: C, 42·9; H, 3·6; N, 25·0%.) CO_2 equivalent to 0·39 mol. was evolved after acidification.

The acidified hydrolysate was filtered from some black amorphous material (0·22 g.) and exhaustively extracted with ethyl acetate. The extract was evaporated *in vacuo* to small bulk and fractionally precipitated by the gradual addition of light petroleum. The first fraction (0·13 g.) was mainly insoluble polymerized material; the third fraction (0·19 g.) was an intractable oil; the middle fraction (0·45 g.), on fractional crystallization from ether-light petroleum, gave finally 0·035 g. of pure colourless crystals, m.p. 186°, together with 0·065 g. of less pure crystals. (Found: C, 61·45; H, 6·5. Mol. wt. (Rast) 274, 273; titration equiv. 255. $C_{13}H_{16}O_5$ requires: C, 61·9; H, 6·4%. Mol. wt. 252; equiv., titrating as a monobasic acid, 252.) The *substance* forms colourless needles which are soluble in ether or water but not in light petroleum. Its aqueous solution is acid to litmus. Its ethanol solution gives with ethanolic $FeCl_3$ first a pure blue colour, then green, and changes to red on addition of a little water. It readily sublimes without decomposition in a high vacuum. It gives no reactions indicative of the presence of a carbonyl group.

(2) *Dihydrofuscin.* Dihydrofuscin (1·0 g.) and 2N-NaOH (20 ml.) were used. After 5 hr. hydrolysis 0·73 mol. of acetaldehyde 2:4-dinitrophenylhydrazone separated in the Brady bubbler, and 0·13 mol. of CO_2 was collected in the baryta bubbler. The acidified hydrolysate yielded an ethyl acetate extract, which on evaporation gave a dark gum containing many colourless needles. This was purified by fractionation from ether-light petroleum, a process involving much loss. 0·18 g. of colourless needles was obtained, m.p. 184°, not depressed on admixture with the substance $C_{13}H_{16}O_5$, m.p. 186°, obtained by the alkaline hydrolysis of fuscin. It also gave the same colour reactions.

(3) *Derivatives and breakdown products of the hydrolysis acid,* $C_{13}H_{16}O_5$. (*a*) *Decarboxylation product, m.p.* 141°. The hydrolysis acid, $C_{13}H_{16}O_5$ (4·5 mg.), mixed with copper chromite (2 mg.) was placed in a small L-shaped tube closed at each end and fitted with a side arm. The other arm contained 1 ml. of 0·1N-$Ba(OH)_2$. After evacuation, the side arm was closed and the pyrolysis mixture was heated at 150°. The baryta showed a film of carbonate and heating was continued at 160° for 2·5 hr. After cooling and allowing to stand for several hours, back titration of the baryta showed the absorption of 0·8 mol. of CO_2. A sublimate of almost colourless needles which formed above the melt was collected. The melting point, 141°, was not depressed on admixture with the phenol $C_{12}H_{16}O_3$, m.p. 139–140°, obtained by pyrolysis or decarboxylation of dihydrofuscin (see sections A 2 and B 2). It also gave the same colour reactions with aqueous and ethanolic $FeCl_3$.

(*b*) *Diacetate.* The hydrolysis acid, $C_{13}H_{16}O_5$ (0·05 g.), was acetylated in the usual way with acetic anhydride (0·5 ml.) and anhydrous sodium acetate (0·05 g.). The recovered crude acetate (0·04 g.), m.p. 160–166°, was recrystallized from ether-light petroleum giving colourless sheaves of needles, m.p. 168°. (Found: C, 61·0; H, 6·35. Titration equiv. 326. $C_{17}H_{20}O_7$, i.e. $C_{13}H_{14}O_3(O.CO.CH_3)_2$, requires: C, 60·7; H, 6·0%. Equiv., titrating as a monobasic acid, 336.) The *diacetate* is readily soluble in ethanol or aqueous $NaHCO_3$, and gives no colour with $FeCl_3$.

(*c*) *Dimethyl ether.* The hydrolysis acid is somewhat unstable. It was therefore found preferable to isolate it for subsequent degradation experiments as a dimethyl ether. Methylation with ethereal diazomethane proved to be difficult and incomplete. The following procedure was satisfactory, the hydrolysis and methylation being carried out in an atmosphere of N_2. A solution of dihydrofuscin (15 g.) in 2N-NaOH (650 ml.) was boiled for 14 hr. Acetaldehyde 2:4-dinitrophenylhydrazone (6·7 g.) was precipitated in the Brady bubblers. The cooled reaction mixture was methylated with dimethyl sulphate (90 ml.) and aqueous NaOH (54 g. in 240 ml. water) added alternately in portions with vigorous stirring, first at room temperature and then at boiling point. The methylation was continued until a sample of the methylated product no longer gave a purple colour with $FeCl_3$. A further 10 ml. of 10N-NaOH was then added and the solution was boiled for 2 hr. to complete saponification.

The cooled methylation mixture was acidified with HCl to congo red and extracted with ether (5 × 500 ml.). The ether extract, on evaporation to 30 ml., yielded the *dimethyl ether* in colourless plates, wt. 8·6 g., m.p. 167°, and a further 0·75 g. from the mother liquors evaporated to dryness and redissolved in dry ether. (Found: C, 64·1; H, 7·3; CH_3O, 21·9; titration equiv. 283·5. $C_{15}H_{20}O_5$, i.e. $C_{13}H_{14}O_3(OCH_3)_2$, requires: C, 64·3; H, 7·2; $2CH_3O$, 22·1%; equiv., titrating as a monobasic acid, 280.) The dimethyl ether may be crystallized from boiling water, or ethanol (1 vol.) + hot water (8 vols.). It gives no colour with $FeCl_3$.

(*d*) *Oxidation of the dimethyl ether with alkaline* $KMnO_4$. The dimethyl ether (14·2 g., m.p. 167°) was dissolved in N-NaOH (80 ml.) and water (100 ml.). Aqueous 10% (w/v) $KMnO_4$ solution was gradually added with constant stirring.

The first portions were readily decolorized in the cold. When approximately 300 ml. had been added, heating on the water bath was commenced. One litre, equivalent to 100 g. $KMnO_4$, was added in all in the course of 2 working days. The hot, pale yellowish-green solution was separated by filtration from the MnO_2 which was thoroughly extracted with hot water. The combined filtrate and washings, acidified with HCl to congo red, were evaporated *in vacuo* to approximately 200 ml. On chilling overnight, colourless crystals, fraction A (4·9 g.), consisting of a mixture of large plates (oxalic acid) and needles, were separated by filtration from the mother liquor, B.

Fraction A was dissolved in N-NaOH (40 ml.) and acidified to litmus with 2 N-HCl (5 ml.). Colourless needles of *oxidation product I* separated (wt. 0·21 g., m.p. 158°). The filtrate from these crystals, when made alkaline with NH_3 and treated with excess $CaCl_2$, yielded a copious precipitate of calcium oxalate.

Mother liquor B, on similar treatment with NH_3 and $CaCl_2$, yielded a further copious precipitate of calcium oxalate, the filtrate from which was strongly acidified with HCl and exhaustively extracted with ether. Removal of the solvent yielded 7·06 g. of yellow syrup which was dissolved in dry ether (30 ml.). Colourless crystals (0·65 g.) quickly separated and a further 0·65 g. of the same compound was obtained on concentrating and chilling the ether solution. This *oxidation product II*, wt. 1·30 g., melted at 187° with gas evolution, then reset while still hot and remelted on further heating at 224°. The ether mother liquors from oxidation product II were dried and the oily residue (4·66 g.) was dissolved in boiling benzene (150 ml.). On cooling, colourless silky needles separated as a suspension in the benzene together with a small amount of oil adhering to the surface of the flask. The crystals were filtered off without disturbing the oil, washed with benzene and dried. They constitute *oxidation product III*, wt. 2·6 g., m.p. 153–155°.

The benzene mother liquors from oxidation product III together with the oil were evaporated to dryness *in vacuo*. The residue (1·13 g.), oily at first, slowly partially crystallized. It was stirred with benzene (3 ml.) which dissolved the oil. The residual crystals constitute *oxidation product IV*, wt. 0·035 g., m.p. 79°. The benzene mother liquors from oxidation product IV were freed from solvent. The residue was dissolved in water and converted into the Zn salt by heating with $ZnCO_3$. There was finally obtained by fractional crystallization from water 0·074 g. of the Zn salt of oxidation product IV.

Oxidation product I, m.p. 158°. This *substance* is readily purified by crystallization from ether or by acidification of an aqueous solution of its Na salt. It forms colourless needles, m.p. 158°. (Found: C, 58·4, 58·6; H, 5·4, 5·7; CH_3O, 23·0; active hydrogen in pyridine, 0·37; titration equiv. either hot or cold 264, 266. $C_{13}H_{14}O_6$ requires: C, 58·6; H, 5·3; $2CH_3O$, 23·3; 1 active hydrogen, 0·38%; equiv., titrating as a monobasic acid, 266.) The substance gives no colour with $FeCl_3$ either in water or ethanol, and no precipitate with Brady's reagent.

Oxidation product II, m.p. 187°, *remelting at* 224°. This *substance* was purified by crystallization from dry ether. It forms colourless crystals, m.p. 187°, with evolution of gas, resetting at once on further heating and remelting at 224°. On cooling the melt and reheating, the melting point remains unchanged. (Found: C, 51·3; H, 4·9; CH_3O, 17·4; *C*-CH_3, 11·4; active H in pyridine, 0·82; titration equiv. 113. $C_{15}H_{16}O_{10}$ requires: C, 50·6; H, 4·5; $2CH_3O$, 17·4; 3(*C*-CH_3), 12·5; 3 active H, 0·82%; equiv., titrating as a tribasic acid, 119.) The acid is readily soluble in water or ethanol and slightly soluble in dry ether. It gives no colour reaction with $FeCl_3$, and no precipitate with Brady's reagent. The residue from a macro-Zeisel methoxyl estimation was diluted with water, extracted with ether, the ether extract shaken with $Na_2S_2O_3$ to remove I_2 and evaporated, yielding a small amount of crystals, m.p. 244°, an ethanolic solution of which gave a green $FeCl_3$ reaction, unchanged on the addition of water.

Silver salt. A quantity of oxidation product II dissolved in a minimum of water was made slightly alkaline with NH_3 and precipitated with a slight excess of 0·1 N-$AgNO_3$. The *silver salt* formed a colourless amorphous precipitate which was carefully washed and dried to constant weight. (Found: C, 26·4; H, 2·3; Ag, 48·8. $C_{15}H_{13}O_{10}Ag_3$ requires: C, 26·6; H, 1·9; Ag, 47·8%.)

Tri-p-bromophenacyl ester. Oxidation product II (0·107 g.) neutralized with N-NaOH (0·97 ml.) was mixed with ethanol (3 ml.), water (1 ml.) and *p*-bromophenacyl bromide (0·26 g.). The mixture was refluxed for 2 hr. The crude ester (0·20 g.) which separated was crystallized from acetone-light petroleum. The *tri-p-bromophenacyl ester* formed colourless fine needles, m.p. 96–97°. (Found: C, 49·45; H, 3·5; Br, 23·8. $C_{39}H_{31}O_{13}Br_3$, i.e. tri-ester of $C_{15}H_{16}O_{10}$, requires: C, 49·4; H, 3·3; Br, 25·3%.)

Anhydride. When oxidation product II (53·9 mg.) was heated in a current of dry N_2 at 195–200° for 0·5 hr. water (3·4 mg. = 6·3%) was evolved. There was no evolution of CO_2 or CO, which were tested for by bubbling the N_2, after absorption of water in $CaCl_2$, first through $Ba(OH)_2$ and then dilute aqueous $PdCl_2$. During the heating a lemon-yellow sublimate was formed. The whole of the heated material was suspended in dry ether (5 ml.) and the undissolved crystals (23 mg.) were separated and dried. The *anhydride* thus obtained formed colourless needles, m.p. 232°. The anhydride may also be prepared by subliming the acid at 160–170°/1 mm. It forms a colourless sublimate resembling snow, m.p. 232°. (Found: C, 53·0, 53·65; H, 3·8, 4·0; CH_3O, 17·7. $C_{15}H_{14}O_9$ requires: C, 53·25; H, 4·2; $2CH_3O$, 18·3%.) The anhydride (10·4 mg.) behaved in a characteristic fashion on titration with aqueous 0·1 N-NaOH to phenolphthalein. NaOH (0·20 ml.) was neutralized rapidly in the cold and a further 0·68 ml. more slowly on heating before a permanent end point was reached. (Found: titration equiv. 118. $C_{15}H_{14}O_9$, titrating as a tribasic acid, requires 113.)

Oxidation product III, m.p. 167°. The crude product (2·6 g., m.p. 153–155°) was recrystallized from benzene (700 ml.) giving colourless needles with a constant m.p. of 167°. (Found: C, 55·6; H, 5·5; CH_3O, 18·2; *C*-CH_3, 5·66; active H in anisole, 0·64, in pyridine, 1·03; titration equiv. 168, 160. $C_{15}H_{18}O_8$ requires: C, 55·2; H, 5·6; $2CH_3O$, 19·0; *C*-CH_3, 1 group, 4·6, 2 groups 9·2; active H, 2H, 0·61, 3H, 0·91%; equiv., titrating as a dibasic acid, 163.) The acid is readily soluble in cold water, ethanol, acetone, ether and hot benzene; slightly soluble in cold benzene, and insoluble in light petroleum. Its aqueous solution gives a yellow colour with aqueous $FeCl_3$; no precipitate with Brady's reagent; no colour with azobenzenephenylhydrazinesulphonic acid or with ammonium molybdate; bromine water is rapidly decolorized with the formation of a white precipitate (see below). The acid completely resisted attempts to reduce it in aqueous ethanol either with Adams's PtO_2 catalyst or with a Pd-$BaSO_4$ catalyst, and was recovered unchanged. The only products, apart from oils, recovered by ozonization of the

acid in aqueous solution were oxalic acid and an at present uncharacterized 2:4-dinitrophenylhydrazone.

Di-p-bromophenacyl ester. This *ester* was prepared in a similar way to that of oxidation product II. It was recrystallized from hot ethanol giving colourless crystals, m.p. 170°. (Found: C, 51·2; H, 3·8; Br, 21·3. $C_{31}H_{28}O_{10}Br_2$, i.e. the di-ester of $C_{15}H_{18}O_8$, requires: C, 51·7; H, 3·9; Br, 22·2%.)

Demethylation product. This *nor-derivative* was prepared from the residues from a macro-Zeisel methoxyl estimation as described for oxidation product II. The ether residue, an oil, wt. 0·1 g., from 0·1011 g. of oxidation product III, was dissolved in ethanol (2 ml.) and mixed with light petroleum to give a faint turbidity. Clusters of colourless needles, wt. 29 mg., m.p. 258° (decomp.), were obtained. (Found: C, 52·8; H, 5·8; active H in anisole, 1·43; titration equiv. 148. $C_{13}H_{14}O_8$ requires: C, 52·4; H, 4·7; 4 active H, 1·34%; equiv., titrating as a dibasic acid, 149.) The nor-acid is readily soluble in water, ethanol, acetone and ether, but insoluble in light petroleum. Its aqueous solution gives a blue colour with a purple tinge with aqueous $FeCl_3$; no precipitate with Brady's reagent and no colour with azobenzenephenylhydrazinesulphonic acid; an orange-yellow colour slowly fading to yellow with ammonium molybdate.

Action of bromine water. A solution of the acid (0·1 g.) in water (10 ml.) was treated with saturated bromine water (4·5 ml.) added in small portions in the course of 0·5 hr. Decolorization was almost immediate in the early stages. Colourless needles quickly separated, wt. 52 mg., m.p. 192° (resetting on cooling). The *bromo compound* may be purified by crystallization from hot water, or preferably by sublimation in a high vacuum at 125–145°. The melting point remains unchanged. (Found: C, 45·5; H, 3·8; Br, 23·2; CH_3O, 17·5. $C_{13}H_{13}O_6Br$ requires: C, 45·2; H, 3·8; Br, 23·2; $2CH_3O$, 18·0%.) 12·4 mg. in ethanol (2 ml.) required 0·38 ml. 0·1 N-NaOH for neutralization to phenolphthalein in the cold. The end point was sharp and was unchanged on heating. $C_{13}H_{13}O_6Br$, titrating as a monobasic acid requires 0·36 ml. 0·1 N-NaOH.

Action of conc. HNO_3. The acid (0·1 g.) was mixed with cold conc. HNO_3 (2 ml.). It dissolved immediately and brown fumes were evolved. After holding the solution at room temperature for 3 days, it was mixed with water (4 ml.). Colourless needles (50 mg.) separated, m.p. 135°. The *nitro compound* is only slightly soluble in cold water. It dissolves more readily in hot water, but does not crystallize on cooling except on the addition of a little dil. HNO_3. It is best recrystallized by dissolving in the theoretical amount of alkali and acidifying. The melting point remains unchanged. (Found: C, 49·3; H, 4·2; N, 4·7; titration equiv. 325. $C_{13}H_{13}O_8N$ requires: C, 50·2; H, 4·2; N, 4·5%; equiv., titrating as a monobasic acid, 311.)

Oxidation product IV, m.p. 79°. This substance was identified as α-hydroxy*iso*butyric acid. (Found: C, 46·2; H, 8·0; titration equiv. 106. Calc. for $C_4H_8O_3$: C, 46·2; H, 7·7%; equiv., titrating as a monobasic acid, 104.) Its melting point was not depressed on admixture with an authentic specimen of α-hydroxy*iso*butyric acid, m.p. 79°. The Zn salt of oxidation product IV was isolated as colourless shining plates from water, and was shown to be zinc α-hydroxy*iso*butyrate. (Found: on specimen dried in a high vacuum to constant weight at 100°: C, 35·7; H, 5·8; Zn, 23·9. Calc. for $(C_4H_7O_3)_2Zn$: C, 35·4; H, 5·2; Zn, 24·1%.) It had the same properties, crystal shape etc., as an authentic specimen of zinc α-hydroxy*iso*butyrate.

F. *By ozonolysis of fuscin*

Ozonized O_2 was bubbled through a solution of fuscin (1 g.) in $CHCl_3$ (100 ml.), cooled in ice, until the solution was almost colourless (2 hr.). The solution was mixed with water (100 ml.) and warmed with thorough shaking on the water bath. The separated cooled $CHCl_3$ solution was washed with water (100 ml.) and the $CHCl_3$ was removed by evaporation *in vacuo* leaving a crystalline residue (0·93 g.) which was purified by crystallization from 50% (v/v) aqueous ethanol, giving colourless platelets, m.p. 222–224° (decomp.), resetting on cooling and then remelting at 234°. (Found: C, 60·0; H, 5·8. $C_{14}H_{16}O_6$ requires: C, 60·0; H, 5·75%.) The *substance* is fairly soluble in $CHCl_3$ and readily soluble in ethanol. Its ethanolic solution gives no precipitate with Brady's reagent and no colour with $FeCl_3$, even after diluting with water.

In a repeat experiment carried out by Dr E. N. Morgan CO_2-free ozonized O_2 was bubbled through a solution of fuscin (0·5 g.) in $CHCl_3$ (50 ml.), cooled in ice, for 2·5 hr., when the original orange colour had changed to pale yellow. The effluent gases were scrubbed first through distilled water and then through 0·2 N-$Ba(OH)_2$. All connexions were made with plastic tubing which, unlike ordinary rubber tubing, is resistant to ozone. CO_2, corrected for blanks, equivalent to 76·6% of 1 mol. was evolved. Water (50 ml.) was now added to the ozonized $CHCl_3$ solution and CO_2-free O_2 was bubbled through for 2·5 hr., the mixture being held at room temperature. A further amount of CO_2, equiv. to 23·4% of 1 mol., was absorbed in a fresh baryta bubbler. Hence 1 mol. of fuscin $C_{15}H_{16}O_5$, on ozonolysis, yields 1 mol. of CO_2 and 1 mol. of $C_{14}H_{16}O_6$ according to the equation

$$C_{15}H_{16}O_5 + O_2 \rightarrow C_{14}H_{16}O_6 + CO_2.$$

Prolonged ozonolysis of fuscin (0·5 g.) in $CHCl_3$ solution (40 ml.) led to extensive breakdown. The main product isolated was an uncrystallizable oil (0·38 g.) together with acetaldehyde (20 mg. of its 2:4-dinitrophenylhydrazone), acetic acid (about 80% of 1 equiv.) and considerable quantities of oxalic acid.

Methylation of the ozonolysis product $C_{14}H_{16}O_6$. The product (0·30 g.), suspended in dry ether (5 ml.) was treated with excess of ethereal diazomethane. The mixture was shaken and when solution was complete the solvent was removed. The residual dried methylated product (0·26 g.), m.p. 122° (decomp.), was recrystallized from ethanol. Colourless hexagonal prisms, m.p. 123° (decomp.). (Found: C, 57·1; H, 6·1; N, 8·4; CH_3O, 9·3. $C_{16}H_{20}O_6N_2$, i.e. $C_{14}H_{16}O_6 + CH_2N_2 + CH_2$, requires: C, 57·1; H, 6·0; N, 8·3; $1CH_3O$, 9·2%.) The *substance*, dissolved in 10 N-H_2SO_4 containing a little $K_2Cr_2O_7$, gives no colour, indicating the probable absence of a pyrazoline ring (Knorr's test).

Conversion of the ozonolysis product $C_{14}H_{16}O_6$ *into a yellow product,* $C_{14}H_{14}O_5$. The colourless ozonolysis product, $C_{14}H_{16}O_6$, is readily and smoothly converted, with the loss of 1 mol. water, into a yellow product, $C_{14}H_{14}O_5$, by any of the following methods. (*a*) By heating above its m.p., 222–224°. (*b*) By heating with acetic anhydride and sodium acetate. (*c*) By treatment with excess cold aqueous NaOH or cold saturated aqueous $NaHCO_3$, and subsequent acidification in each case. The *yellow product* crystallizes beautifully from ethanol in long silky pure yellow needles, and may be readily sublimed in a high vacuum at 160–180°. M.p. 237° without decomposition, but with some sublimation. (Found: C, 64·15; H, 5·4; active H in pyridine, 0·0; Mol. wt. (Rast) 273.

$C_{14}H_{14}O_5$ requires: C, 64·1; H, 5·4%; mol. wt. 262.) The substance is almost insoluble in water, but dissolves to a yellow solution in hot NaOH, being recovered unchanged on acidification. It is insoluble in aqueous $NaHCO_3$. It is readily soluble in $CHCl_3$ from which it is precipitated in fine crystals by ether or light petroleum. Its ethanolic solution gives no marked colour with ethanolic $FeCl_3$ and no precipitate with Brady's reagent. It gives a clear orange-brown solution in cold conc. H_2SO_4. It resists acetylation with sodium acetate and acetic anhydride, and on treatment with ethereal diazomethane a yellow oil, which could not be obtained crystalline, was formed. Similarly, ozonolysis in $CHCl_3$ afforded an uncrystallizable red gum. Catalytic reduction of its ethyl acetate solution (0·1 g. in 30 ml.) with Adams's PtO_2 catalyst (0·02 g.) resulted in the uptake in 3·5 hr. of 23 ml. H_2. ($C_{14}H_{14}O_5$ absorbing 6H requires 25·7 ml. H_2.) The isolated reduction product was an oil (0·12 g.). Similarly, catalytic reduction of the ethanolic solution of the colourless ozonolysis product, $C_{14}H_{16}O_6$, with $PdCl_2$-norite resulted in the slow uptake of almost 2H in 27 hr. and the formation of a stiff oil.

SUMMARY

An account is given of a number of derivatives and degradation products of fuscin, an antibacterial metabolite of the mould *Oidiodendron fuscum* Robak.

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REFERENCES

Kinney, C. R. & Langlois, D. P. (1931). *J. Amer. chem. Soc.* **53**, 2189.

Michael, S. E. (1948). *Biochem. J.* **43**, 528.

Slotta, K. H. & Müller, J. (1936). *Hoppe-Seyl. Z.* **238**, 14.

Studies in Immunochemistry

9. THE OXIDATION OF THE HUMAN BLOOD-GROUP A SUBSTANCE WITH THE PERIODATE ION

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Apart from the methylation studies of Bray, Henry & Stacey (1946) few structural investigations have been carried out on the blood-group A substance. These workers isolated and identified 2:3:4-trimethyl-α-methyl-L-fucoside and 3:4:6-trimethyl-α-methylglucosaminide hydrochloride in the esterified products from acid hydrolysis of a fully methylated A substance derived from pepsin, thus indicating that both fucose and *N*-acetylglucosamine are present as non-reducing end groups in the alkali-stable residues examined. A methylated derivative of D-mannose was also identified, but according to other investigators (Bendich, Kabat & Bezer, 1947; Partridge, 1948*b*; Aminoff, Morgan & Watkins, 1950) mannose is not present in the A substance. The behaviour of the A substance during mild hydrolysis with alkali (Morgan, 1946) and with acid (Kabat, Baer, Bezer & Knaub, 1948; Baer, Dische & Kabat, 1948; Aminoff, Morgan & Watkins, 1948, 1950) has provided additional information regarding the *N*-acetylhexosamine and fucose components and the roles they play in the different serological activities associated with the blood-group substances.

Oxidation of the A substance with periodate offers an independent and more specific approach to the determination of the structure of this important biological material. Periodic acid is a selective oxidizing agent which breaks the C—C linkage in substances containing either two adjacent unsubstituted hydroxyl groups or an unsubstituted hydroxyl group adjacent to a primary or secondary amino group. The products of oxidation are aldehydes, acids and, with hydroxyamino derivatives, ammonia. The reactions, under suitable conditions, are practically quantitative. The application of oxidation with periodate to structural studies in the field of carbohydrate chemistry has been reviewed by Owen (1943), Jackson (1944), and Jones (1946), and further discussion here is unnecessary.

The investigation of the oxidation of the A substance with periodate was approached along the following lines: (1) studies on the lability of the serological properties during oxidation; (2) determination of the amount of periodate reduced and the rate of reduction by the A substance under different conditions; (3) qualitative and quantitative analyses of

the material after oxidation to determine which of the constituent molecules are involved; and (4) analysis of the simple products liberated during the oxidation. A preliminary communication on this subject has already appeared (Aminoff & Morgan, 1949).

EXPERIMENTAL

The preparations and properties of the A substance used in the oxidation studies are described by Aminoff *et al.* (1950). Most of the oxidations were carried out at room temperature, and the periodate content of the reaction mixture was determined by titrating, with standard arsenite, the I_2 liberated on addition of KI (Jackson, 1944).

Rate of inactivation of A substance at different pH *values*

The A substance was oxidized with HIO_4 at room temperature at pH values between 5 and 10 using acetate, phosphate, bicarbonate and borate buffers. The initial concentration of A substance was 0·1%, of periodate 0·005 M and of

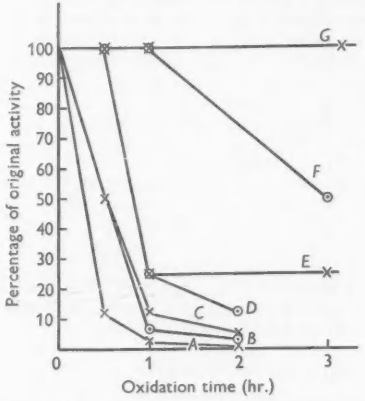


Fig. 1. The effect of oxidation of the A substance with periodate at different pH values as determined by the capacity of the A substance to inhibit the iso-agglutination of A cells by anti-A serum. (*A*) pH 5, acetate; (*B*) pH 6, acetate; (*C*) periodic acid alone; (*D*) pH 8, phosphate; (*E*) pH 8, borate; (*F*) pH 9, (*a*) borate and (*b*) bicarbonate (*G*) pH 10, borate.

buffer 0·075–0·1 M. The immediate and complete reduction of the excess periodate in 0·5 ml. quantities of the reaction solution, withdrawn at pre-arranged intervals, was accomplished by addition of 0·2 ml. of 20% (v/v) glycerol. The resulting solutions were then diluted with 0·85% NaCl to make a final dilution of 1:5000 of the A substance originally present, and the capacity of the material to inhibit iso-agglutination was determined by the standard method (cf. Morgan & King, 1943). The property of inhibiting the agglutination of A red cells by anti-A serum is rapidly lost, and when this specific activity, expressed as a percentage of the original value, is plotted against time a series of curves is obtained (Fig. 1). It is seen that with one exception the rate of inactivation falls with rise in pH. The system containing free periodic acid, curve *C*, in the absence of buffer, behaves as though its pH were between 6 and 7, which is surprising in view of the fact that periodic acid has a pH of 2·5 under the conditions used.

Rate of reduction of periodate by A substance

Reaction at pH 5. A 0·1% solution of A substance was made up to contain 0·005 M-HIO_4 and 0·1 M-acetate buffer at pH 5·0. At appropriate intervals 2 ml. portions of the solution were withdrawn and added to conical flasks which contained 0·1 N-sodium arsenite (0·5 ml.) and 9% (w/v) $NaHCO_3$ (0·5 ml.). The walls of the flask were washed down with 1 ml. of water and 20% (w/v) KI (0·1 ml.) was immediately added. After standing at room temperature for 15 min. the excess arsenite, not oxidized by the periodate, was titrated with 0·01 N-I_2 solution using 0·5 ml. of 1% starch solution as indicator.

At the same time as each specimen was removed for titration, 0·4 ml. of the reaction mixture was also withdrawn and added to 1·6 ml. of glycerol-NaCl solution (0·2 ml. 20% (v/v) glycerol with 1·4 ml. 0·85% NaCl) to give a 1:5000 dilution of the A substance originally present, for the determination of serological activity. The solutions were kept at 0° until the experiment was completed and then assayed for the capacity of the material to inhibit (*a*) the agglutination of A cells, and (*b*) the haemolysis of sheep cells by anti-A serum.

Owing to the rapidity of reduction of periodate during the initial stages of the reaction, the periodate content at zero time was determined on a solution which contained all the reagents without A substance. This solution was also used to check the pH of the reaction mixture. Fig. 2, obtained by

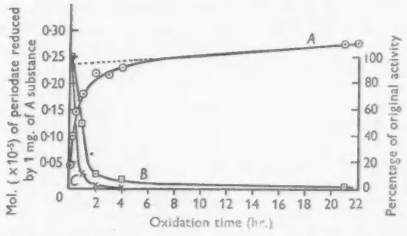


Fig. 2. The oxidation of the A substance at pH 5. Rate of reduction of periodate by the A substance (*A*). The resulting inactivation as determined by the capacity of the material to inhibit the haemolysis of sheep cells (*B*), and the agglutination of A cells (*C*), by anti-A serum.

plotting the periodate reduced against the time of oxidation, shows that the reaction takes place in two distinct phases. An initial rapid reaction which is essentially complete after 4 hr. and a second phase in which the reduction still continues, but at a much reduced rate. The second phase is not due to instability of the periodate or to the oxidation of acetate present, since the control solution showed no fall in periodate content over a period of 44 hr. A correction for the over-oxidation can be obtained by simple extrapolation to zero time. The analytical results reveal that 1 mg. of A substance reduces $0·235 \times 10^{-5}$ g.mol. of HIO_4 and hence 427 g. of A substance reduce 1 g.mol. of HIO_4. A similar value, 370 g. of A substance/1 g.mol. of HIO_4, was obtained with

preparation III (Aminoff *et al.* 1950). The power of the A substance to inhibit iso-agglutination falls very rapidly and reaches a value of not more than 1% of the original activity in 3 hr. The capacity of the material to inhibit anti-A sheep-cell haemolysins falls less rapidly and oxidation for 22 hr. is required to bring about inactivation to the same extent.

Reaction at pH 7·3. The reaction was carried out under the same conditions as described for the oxidation at pH 5·0. The HIO_4 concentration was, however, doubled as a preliminary experiment indicated that a greater consumption of periodate occurred at pH 7·3. Owing to the low solubility of $NaIO_4$ in the reaction mixture an opalescence developed and frequent shaking was necessary. A 0·1% solution of the A substance was prepared in a solution of 0·01 M periodate and 0·04 N-$NaHCO_3$ at pH 7·3. At intervals 1 ml. samples were withdrawn and titrated against standard I_2 as described above.

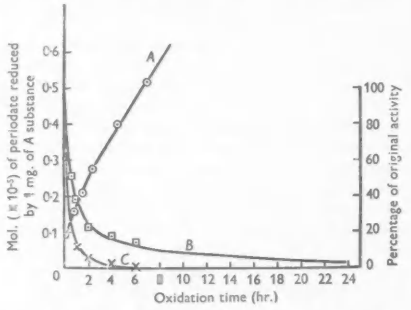


Fig. 3. The oxidation of the A substance at pH 7·3. Rate of reduction of periodate by the A substance (*A*). The resulting inactivation as determined by the capacity of the A substance to inhibit the haemolysis of sheep cells (*B*), and the agglutination of A cells (*C*), by an anti-A serum.

Samples (0·4 ml.) were also withdrawn for the serological titrations. The oxidation curve, Fig. 3, shows a more extensive and continuous reduction of periodate than was found for the oxidation at pH 5. There was only a slight indication of the existence of a point of inflexion and this occurred after about 2 hr. The examination of a sample of the reaction mixture at 23 hr. showed the complete reduction of all the periodate present, whereas the titration results with the control solution indicated that periodate was perfectly stable under these conditions. The fall in the serological activities is not as rapid as at pH 5.

Rate of oxidation in the presence of borate. In the presence of boric acid at pH 2·4 the oxidation of A substance was essentially complete in 3 hr. and 1 g. mol. of periodate was reduced by 294 g. of A substance (preparation III). The oxidation was also carried out in $NaHCO_3$ solution at pH 7·5 and with $NaHCO_3$-borate mixture at the same pH. The course of oxidation was identical in these two buffer systems and was not complete after 72 hr.

Re-oxidation at pH 5·0 *and* 7·5 *of A substance oxidized at* pH 5. The solution of A substance obtained after oxidation at pH 5 was dialysed in a thoroughly washed cellophan tube. A preliminary experiment had shown that the material obtained after drying the oxidized product from the frozen state was largely insoluble in water. To determine the concentration of the main bulk of the solution only a portion of the oxidized material was therefore taken to dryness. The concentration of the solution was found to be 0·476% with respect to the oxidized A substance. The recovery was 89%, no allowance being made for the weight of diffusible formic acid and formaldehyde, which together would have been of the order of 4% (see below).

Re-treatment of this solution with more periodate at pH 5·0 caused no further oxidation to occur (Fig. 6, curve *C*). At pH 7·5, however, there was a steady reduction of the periodate (Fig. 6, curve *D*). In one particular experiment, after 21 hr. at pH 5, 1 mg. of the oxidized A substance had reduced only $0{\cdot}05 \times 10^{-5}$ g.mol. of HIO_4, whereas at pH 7·3 the corresponding value was $0{\cdot}895 \times 10^{-5}$ g.mol. and after 45 hr. $1{\cdot}25 \times 10^{-5}$ g.mol. had been reduced.

Re-oxidation at pH 5 *and* 8 *of A substance oxidized at* pH 7·5. The product from A substance after oxidation (48 hr., pH 7·5) and dialysis was mixed with 0·008 M-HIO_4 in (*a*) 0·25 M-acetate buffer pH 5, and (*b*) 0·04 N-$NaHCO_3$, pH 8. The course of oxidation was followed in the usual manner. Since the concentration of the oxidized A substance was not exactly known in this experiment, the results are expressed in terms of molarity of the solution with respect to the periodate.

The curves for the re-oxidation at pH 5 and 8 of material already oxidized at pH 7·5 are very similar (Fig. 4). The reaction in each instance approaches completion in about 24 hr., exclusive of the original 48 hr. oxidation at pH 7·5.

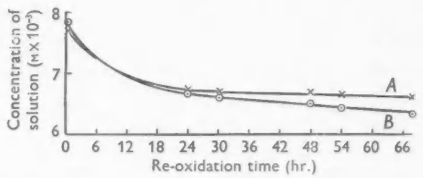


Fig. 4. Comparison of the rate of reduction of periodate by the A substance at (*A*) pH 5 and (*B*) pH 8 after initial oxidation with periodate at pH 7·5.

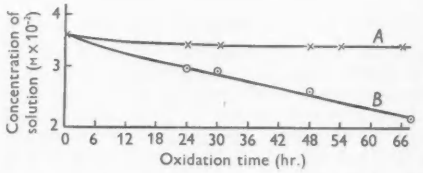


Fig. 5. Comparison of the rate of reduction of periodate by the A substance at (*A*) pH 5 and (*B*) pH 8 after an initial treatment with 0·5 N-$NaHCO_3$.

The effect of initial treatment with $NaHCO_3$ *on the course of oxidation.* In view of the known alkali-lability of the A substance (Morgan & King, 1943; Morgan, 1946) an experiment was carried out with undegraded A substance which had been exposed to 0·5 N-$NaHCO_3$ for 24 hr. at about 16° to determine whether the more extensive oxidation at pH 7·5 was due to the dissociating effect of the $NaHCO_3$. The results (Fig. 5) indicate that initial treatment with bicarbonate in no way influences the course of the subsequent oxidation at pH 5 (curve *A*) and pH 8 (curve *B*) (cf. Figs. 2*A* and 3*A*).

In order to determine whether the material oxidized at pH 5·0 is more labile to alkali than the unoxidized A substance some of the material oxidized at pH 5·0 was treated with $NaHCO_3$ at pH 9·4 for 24 hr. Subsequently, half the solution was adjusted to pH 5 with acetic acid and the HIO_4 added, whilst the addition of HIO_4 to the remainder of the alkali-treated material resulted in a reaction mixture with a

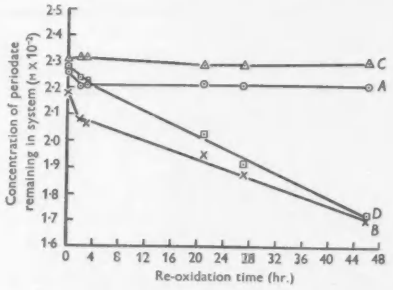


Fig. 6. Course of re-oxidation with periodate (at pH 5·0 or 7·5) of oxidized A substance (preparation III) with and without intermediate treatment with $NaHCO_3$ (pH 9·4; 24 hr.). *A*, re-oxidation at pH 5 after treatment with $NaHCO_3$ at pH 9·4; *B*, re-oxidation at pH 7·5 after treatment with $NaHCO_3$ at pH 9·4; *C*, re-oxidation at pH 5 without the initial treatment with $NaHCO_3$ at pH 9·4; *D*, re-oxidation at pH 7·5 without the initial treatment with $NaHCO_3$ at pH 9·4.

pH value of 7·5. The course of the re-oxidation at pH 5 and 7·5 of (*a*) material oxidized at pH 5·0 and then treated with $NaHCO_3$ for 24 hr. (at pH 9·4) and (*b*) some of the material oxidized at pH 5·0 without treatment with the $NaHCO_3$, was followed in the usual manner and the results are shown in Fig. 6. The results indicate that the more extensive oxidation at pH 7·5 is not due to the greater alkali lability of the primary oxidation products of the A substance.

Qualitative and quantitative examination of oxidized A substance

Unless otherwise stated, the tests were carried out on a 0·476% solution of the product from A substance (preparation III) oxidized at pH 5·0, 24 hr., which had been thoroughly dialysed.

Qualitative tests. The oxidized material readily restored the colour to SO_2-decolorized fuchsin (Schiff's reagent). The unoxidized A substance did not restore the colour. Oxidation, therefore, gives rise to the formation of an indiffusible aldehyde. A Molisch test with the oxidized A substance yields an intense bluish coloured boundary layer, with no trace of pink coloration; whereas, tested under the same conditions, unoxidized A substance gives a bright-red interfacial layer, and A substance previously added to the oxidized material imparts a reddish tint to the blue coloration. It may be concluded, therefore, that galactose and fucose, both of which readily give a positive Molisch reaction, are not present in the oxidized A substance.

Chromatographic analysis of the products of acid hydrolysis of the oxidized A substance. Oxidized and unoxidized A substance (5 mg.) were hydrolysed for 16 hr. with (*a*) N-acetic acid, (*b*) 0·5N-HCl and (*c*) 6N-HCl. The unoxidized A substance gave more humin during the hydrolysis than did the oxidized material and the N-acetic acid hydrolysis products of the oxidized A substance dissolved less readily than the other products in water.

The hydrolysis products arising from the unoxidized and oxidized A substance were analysed simultaneously, and the results are strictly comparable. No difference could be detected in the amino-acid distribution of the materials when run in two directions on paper with collidine (54 hr.) and phenol-ammonia-coal gas (24 hr.) as solvent systems. Both hexosamines were present in the oxidized A substance as demonstrated by the ninhydrin, ammoniacal silver and the hexosamine tests. Quantitatively, however, the spot attributed to chondrosamine was less intense after oxidation of the A substance. Fucose was absent from the hydrolysis products of the oxidized A substance. The hydrolysates (0·5N-HCl, 16 hr.) of A substance oxidized at pH 5 (24 hr.) and at pH 7·5 (48 hr.), when run in *n*-butanol-water containing 3% acetic acid in the lower aqueous layer (Aminoff *et al.* 1950) were found to contain only a trace of galactose.

The total fucose content of the A substance. The colorimetric method of Dische & Shettles (1948) was employed using a Hilger Uvispek spectrophotometer. This method determines the total fucose content, free and combined, whereas the method involving the oxidation with periodate (Aminoff *et al.* 1950) only measures the fucose residues with the hydroxyl groups on C atoms 4 and 5 unsubstituted, e.g. free fucose as liberated during mild acid hydrolysis. The material was heated at 100° for 10 min. with the H_2SO_4 reagent, and the colour developed was measured 3–4 hr. after the addition of the cysteine hydrochloride (3%). The absorption curves of

Table 1. *Total fucose content of the A substance and some of its degradation products as determined by the colorimetric method of Dische & Shettles* (1948)

Substance	Percentage of original A substance	Fucose: Percentage	Fucose: Percentage of original A substance
A substance	—	18·5*	—
A substance 16 hr. N-Acetic acid hydrolysate:			
(*a*) Diffusate	38	28·2	10·9
(*b*) Indiffusible material	62	12·4	7·7
A substance (preparation III)	—	18·3	—
A substance oxidized with periodate at pH 5 for 24 hr.	—	0·82	—
A substance oxidized with hypoiodite	—	16·3	—

* A preparation of A substance obtained from cyst fluid no. 122 gave 18·4% fucose.

various concentrations of L-fucose, the A substance and an artificial mixture of N-acetylglucosamine, N-acetylchondrosamine, D-galactose and L-fucose, in the proportions believed to be present in the A substance, were plotted over the range 370–460 mμ. The maximum absorption was found to be at 400 mμ. on this instrument and not at 396 mμ. as quoted by Dische & Shettles (1948) (cf. Dische, Shettles & Osnos, 1949). The fucose content was, therefore, calculated on the basis of the difference in the extinctions at 400 and 430 mμ. The results are given in Table 1 and show that the best preparation of A substance contains 18·5% fucose. Preparation III (Aminoff *et al.* 1950) gave a lower value, 18·3%. The results also indicate that all the fucose in the A substance is destroyed by oxidation with periodate, and that the non-carbohydrate constituents in the complex do not interfere with the fucose determination. In contrast, the A substance oxidized with sodium hypoiodite retains its fucose residues largely unchanged.

N-Acetylhexosamine and hexosamine colour reactions. A substance (preparation III) gave a colour equivalent to 10·4% *N*-acetylglucosamine (Morgan & Elson, 1934), whereas the material after oxidation for 24 hr. at pH 5 gave a colour equivalent to 9·5% *N*-acetylglucosamine. There is, therefore, little change in this property as a result of oxidation. Nevertheless, after acid hydrolysis, the oxidation products are found to contain less free hexosamine base (Table 2), and the nature of the distribution of the hexosamines in the undegraded A substance and its oxidation products are given below.

Quantitative determination of the distribution of hexosamines. Portions (5 μl.) of the hydrolysates (6N-HCl, 3 hr., 100°) of the A substance before and after oxidation, or of an artificial mixture of glucosamine and chondrosamine, each of which contained about 50 μg. of total amino-sugar hydrochloride, were placed 4 cm. apart on a straight line 6 cm. from the top edge of a sheet (32 × 56 cm.) of Whatman no. 1 paper. The sheet was irrigated for 48 hr. with collidine saturated with water. The collidine had been purified according to the technique described by Partridge (1948*a*) and subsequently carefully fractionated through a Fenske column at 15 mm. pressure to give a colourless product. The paper was then dried overnight at room temperature and cut into strips, each corresponding to the path of the substances derived from a single spot. Alternate strips were sprayed with the hexosamine reagents (Partridge, 1948*a*) to locate the positions of glucosamine and chondrosamine. The corresponding areas in the unsprayed strips were cut out and eluted with water in a covered glass chamber as shown in Fig. 7. Approximately 1·5 ml. of the eluate was collected directly into the test tubes used for the determination of hexosamines and calibrated at the 2 and 10 ml. levels. The funnel and walls of the tube were then washed down with water to yield a final volume of 2 ml. The hexosamine in the eluate was determined by a modification of the procedure of Elson & Morgan (1933).

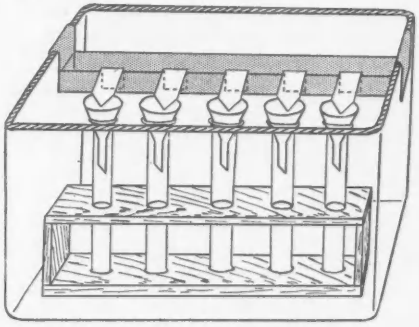


Fig. 7. The glass chamber used for the elution of sugars and hexosamines from the appropriate areas of a paper-strip chromatogram.

The results obtained with an artificial mixture of equal amounts of glucosamine and chondrosamine hydrochlorides and hydrolysates of the undegraded A substance and some of its oxidation products are given in Table 2. It is seen that the

Table 2. *Quantitative distribution of the hexosamines in the A substance and its oxidation products*

Material and treatment	Total hexosamine (base) (%)	Chondrosamine (μg.)	Glucosamine (μg.)	Recovery (%)	Ratio glucosamine/ chondrosamine
a Chondrosamine and glucosamine hydrochlorides (27 μg. of each)	—	11·5±6%	13·5±7·5%	46	1·17
b A substance (preparation III), hydrolysed with 6N-HCl for 16 hr.	32	10·0±10%	8·9±18%	33	0·89
c A substance (preparation III), oxidized at pH 5 for 24 hr. and then hydrolysed with 6N-HCl for 16 hr.	24	6·4±6%	12·5±2%	44	1·95
d A substance hydrolysed with 6N-HCl for 3 hr.	37	7·9±8%	7·8±15%	33	1·01
e A substance oxidized at pH 4·5 and then hydrolysed with 6N-HCl for 3 hr.	27	3·7±13%	7·0±7%	30	1·90
f A substance hydrolysed with 6N-HCl for 3 hr.	37	7·1	7·6	36	1·07
g A substance oxidized at pH 7·5 and then hydrolysed with 6N-HCl for 3 hr.	23	3·9±23%	7·3±8%	22	1·90

recovery of the hexosamines is of the order of 30–50% only. It is known, however, that the free hexosamine bases are unstable in an alkaline medium and this would account for the low recovery obtained with collidine (pH 9·4–10·0) as the solvent. In an acidic medium such as butanol-acetic acid, the recovery of the total hexosamine from the paper was up to 90%, but unfortunately it is only in basic solvents such as collidine and butanol-ammonia that a useful separation of the two hexosamines is achieved (Aminoff & Morgan, 1948). The recovery of the hexosamines with collidine as solvent can be much improved by running the chromatograms at 0°, but the rate of irrigation is greatly reduced owing to the increased viscosity of the solvent.

Despite the obvious limitations of the method for the determination of the total hexosamine, the similar behaviour of glucosamine and chondrosamine under these conditions makes possible the determination of the relative distribution of the two hexosamines. In the undegraded A substance glucosamine and chondrosamine are present in equimolecular proportions. After oxidation at pH 5, however, the hexosamine content of the hydrolysate of the oxidized material falls to about 75% of that of the undegraded A substance and the glucosamine/chondrosamine ratio is approximately 2, thus indicating that the fall in hexosamine content can be attributed entirely to the oxidation of half of the total chondrosamine. Oxidation of the A substance at pH 7·5, under the conditions used for the determination of formaldehyde (see below) results in a product which contains 23% hexosamine and represents only 64% of the total hexosamine in the undegraded A substance. The ratio glucosamine/chondrosamine in the material oxidized at pH 7·5 is again 2, and therefore oxidation at the higher pH involves some of the glucosamine and rather more than half of the chondrosamine in the A substance.

Analysis of the products liberated during oxidation

Determination and characterization of formaldehyde as the dimedone derivative. Preliminary determinations of the formaldehyde produced during the oxidation of the A substance, by steam distillation and titration of the bound bisulphite, indicated an apparently greater production of formaldehyde than that determined by the dimedone method. In view of the findings of Bell (1948) that the distillate from the oxidation products of some methylated hexoses contained bisulphite-binding compounds other than formaldehyde, it was decided to dispense with the distillation and to precipitate the formaldehyde *in situ* following the procedure of Reeves (1941). In order to avoid hydrolysis of the oxidized A substance with the possible liberation of other aldehydes (e.g. glyoxal), the oxidized material was not heated with the dimedone reagents. Nicolet & Shinn (1941*b*) recommend 48–72 hr. standing with the dimedone reagent, whereas Reeves (1941) and Rees (1946) find 16–24 hr. sufficient.

A 2% aqueous solution of the A substance, preparation III (1·0 ml.), was added to 2 ml. of 9% (w/v) $NaHCO_3$ and 1 ml. of 0·5 M-HIO_4 contained in a stoppered tube. After 24 hr. oxidation at room temperature, the following reagents were added with vigorous shaking in the order indicated: N-HCl (3 ml.), approx. *N*-sodium arsenite (2 ml.), M solution of sodium acetate (2 ml.) and 8% dimedone reagent in 95% ethanol (1 ml.). The resulting mixture was well stirred and kept at room temperature for 48 hr. to permit the complete precipitation of the dimedone derivative. The precipitate which formed was collected on a porosity 3 sintered-glass funnel, washed with cold water and dried to constant weight in an electric oven at 60°. The precipitate (*a*) weighed 3·82 mg. More prolonged oxidation, 48 hr., gave a precipitate (*b*) of similar weight 3·72 mg. The reagents alone gave (*c*) 0·18 mg. of precipitate. The product from A substance, oxidized (pH 5, 24 hr.) and dialysed, was treated with periodate and $NaHCO_3$ and immediately HCl, sodium arsenite and the other reagents were added. A precipitate (*d*) slowly developed and increased in amount on long standing. After a week at room temperature the precipitate was voluminous and proved difficult to filter. Drying the material at 60° gave rise to charring and the substance became gummy.

The melting points of the different precipitates were not sharp. After recrystallization from ethanol, in which precipitates *a*, *b* and *c* were readily and *d* sparingly soluble, *a* and *b* gave long needles, m.p. 185° (uncorr.). Precipitate *d* crystallized very poorly and softened slowly with charring between 178 and 196°. The ready solubility of *a*, *b* and *c* in ethanol in contrast to the low solubility of *d* indicated that the weight of the true dimedone derivative of formaldehyde could be obtained as the weight of the precipitate that was soluble in ethanol.

By this simple modification in technique it was shown that 32·83 mg. of the purest A substance, on oxidation for 24 hr. and filtration 20 hr. after the addition of dimedone reagents, gave rise to 5·21 mg. of ethanol-soluble formaldehyde-dimedone derivative of which 0·33 mg. was derived from the reagents. 1 g.mol. of formaldehyde is therefore obtained from 1964 g. of A substance. For preparation III a higher value, 2551 g., was obtained.

After removal, by filtration, of the formaldehyde-dimedone derivative from the oxidation products of the purest A substance the filtrate was dialysed and dried from the frozen state. The hexosamine content and the relative distribution of the glucosamine and chondrosamine residues were determined on a hydrolysate (6 N-HCl for 3 hr.) of the material and the results obtained are given in Table 2, *g*.

After oxidation under the same conditions methyl *N*-acetylglucosaminide gave only a trace of precipitate, whereas *N*-acetylglucosamine gave 93–95% of the theoretical amount of formaldehyde as dimedone derivative (m.p. 186°, uncorr.) identical with the material formed on the addition of formaldehyde to dimedone.

Determination of the acid produced during oxidation. It is only recently that the estimation of the acid produced during oxidation of substances of large molecular weight with periodate has been investigated as a method of end-group assay. Two methods have been suggested to overcome the difficulty of 'over acid production' (*a*) the slow oxidation at room temperature with the sparingly soluble KIO_4 (Halsall, Hirst & Jones, 1947), and (*b*) the more rapid oxidation with $NaIO_4$ at a lower temperature (Potter & Hassid, 1948).

(*a*) Employing the method of Halsall *et al.* (1947). The A substance (2 ml. of 2% solution of preparation III) was treated in a 25 ml. flask with 5 ml. of 0·254 M-HIO_4, which had been brought to pH 4·5 (methyl red) with NaOH, and 4 ml. of 25% (w/v) KCl solution were added. The solution was then made up to 25 ml. with distilled water. Throughout the period of oxidation the mixture was kept in the dark and at room temperature. It was shaken frequently to disperse the crystalline precipitate of KIO_4. Samples (4 ml.) were withdrawn at intervals over a period of 237 hr. and added to flasks containing 0·75 ml. of ethylene glycol (which had

Table 3. *Rate of reduction of periodate and production of acid on oxidation of the A substance with* KIO_4 *and* $NaIO_4$

KIO_4		$NaIO_4$ at 2–3°		
			Weight of A substance (preparation III)	
Period of oxidation (hr.)	Weight of A substance (preparation III) giving 1 g. mol. of acid (g.)	Period of oxidation	Giving 1 g. mol. of acid (g.)	Reducing 1 g. mol. of periodate (g.)
26	1935	7 min.	11840	304
75	1575	185 min.	—	255
170	1215	345 min.	3480	258
215	1030	27 hr.	2190	250
237	1092	47 hr.	1740	235
		70 hr.	1535	229

previously been distilled over NaOH) and kept in the dark for 15 min. The excess periodate slowly dissolved and the resulting solution was titrated against 0·01 N-$Ba(OH)_2$ using methyl red as indicator. A reagent blank (4 ml.) was treated similarly and the appropriate corrections applied. The production of acid had reached a maximum in 215 hr. (Table 3), after which time 1030 g. of A substance would have produced 1 g. equiv. of acid.

(*b*) Oxidation with $NaIO_4$ (Potter & Hassid, 1948); A substance (2 ml. of 2% solution of preparation III) contained in a 25 ml. flask was treated with 5 ml. 0·207 M-HIO_4 brought to pH 4·5 (methyl red) with NaOH and 3·75 ml. of 10% (w/v) NaCl added and made up to 25 ml. with water. The solution was kept in the dark at 2–3°. At intervals samples were withdrawn, added to ethylene glycol as described above and after standing for 1 hr. in the dark, titrated with 0·01 N-$Ba(OH)_2$. At the same time portions of the solution were added to alkaline arsenite solution and the course of the periodate reduction followed in the usual manner. The reduction was practically complete in 3 hr., after which time 255 g. of A substance would have reduced 1 g.mol. of periodate. After that period of oxidation 1535 g. A substance would have liberated 1 g. equiv. of acid. Details of the experimental results are given in Table 3. Under the same conditions 1 g. equiv. of acid would have been liberated by 2120 and 1790 g. of the purest specimen of A substance after 26 and 51 hr., respectively.

After neutralization with $Ba(OH)_2$ the oxidation products of the purest A substance were pooled, thoroughly dialysed and dried from the frozen state. The products of hydrolysis (3 hr., 6 N-HCl at 100°) of this material contained 27% of hexosamine, or 74% of the value (37%) for the undegraded A substance. Quantitative chromatographic analysis of this material gave a glucosamine: chondrosamine ratio of 2:1 (Table 2*e*).

Maltose and lactose behaved similarly when oxidized under the same conditions. The periodate was rapidly reduced, the reaction being complete in about 1 hr. 6 g.mol. of periodate were reduced per g.mol. of disaccharide, instead of the expected 5 g.mol. Again, the rate of acid production was much slower than the rate of reduction of periodate, and ultimately exceeded the theoretical amount of 3 g.mol./g.mol. of disaccharide. The theoretical amount was, however, obtained for both maltose and lactose after a period of oxidation of 25 hr.

Determination of hydroxyamino nitrogen. The method employed was based on that described by Gordon, Martin & Synge (1943). The oxidations were carried out in Conway units and the NH_3 liberated absorbed in boric acid.

Boric acid (1 ml., 2% solution) which contained a mixed bromocresol green-methyl red indicator (Ma & Zuazaga, 1942) was placed in the inner chamber of a Conway unit and the following solutions were added to the outer chamber in the order given: (*a*) the substance to be oxidized contained in 0·5 ml. water; (*b*) 9% (w/v) glycine solution (0·1 ml.); (*c*) N-NaOH (0·25 ml.); (*d*) M-HIO_4 (0·2 ml.); and (*e*) saturated solution of K_2CO_3 (1 ml.). The unit was then immediately sealed, carefully rotated to ensure mixing and kept at room temperature. After the given period of oxidation the contents of the inner chamber were titrated with 0·01 N-HCl to a faint-pink colour. The results are given in Table 4 which also includes the results obtained with several substances that were used for control purposes. It will be seen that ethanolamine gives 94–98% recovery, depending on the time of oxidation. Threonine yields its N quantitatively as NH_3, whereas glucosamine hydrochloride gives only about 70% of its N in this form. *N*-acetylglucosamine reacts slowly and liberates rather less than half of its N as NH_3 in 24 hr. Undegraded A substance gives no NH_3 after 3 hr. oxidation and a trace only after 24 hr., and there is, therefore, no possibility of liberated NH_3 interfering with the determination of the acid produced during the oxidation of the A substance. An acetic acid hydrolysate of A substance gives only 7–8% of the total N as hydroxyamino N. This could arise from (1) *N*-acetylhexosamine, (2) hexosamine or (3) the hydroxyamino-acids, serine or threonine. The acetic acid hydrolysis products of A substance which had previously been oxidized for 24 hr. at pH 5 give more than twice as much NH_3 as the hydrolysate of unoxidized A substance. This result could be attributed to the greater lability of the oxidized A substance to acid hydrolysis, and suggests that some of the more labile linkages involve the hexosamines and/or hydroxyamino-acids. The value obtained for the 6 N-HCl hydrolysis products (Table 4) represents the total hydroxyamino N and amide N. The hydrolysate contained 37% hexosamine (corrected for the high values obtained with 6 N-HCl) and, assuming 68% conversion of the hexosamine N into NH_3, would account for 34% of the 47% N appearing as NH_3 on oxidation. Thus 13% of the total N is due to hydroxyamino acids and amide N. This represents quite a high proportion (34%) of the

Table 4. *Hydroxyamino-nitrogen content of miscellaneous substances as determined by the liberation of ammonia on oxidation with periodate*

Substance	Treatment	Period of oxidation (hr.)	Hydroxyamino N as percentage of total N
Ethanolamine	—	2	94·5
Ethanolamine	—	4	95·0
Ethanolamine	—	24	98·0
Threonine	—	2·5	99·0
Threonine	—	21	100·0
Glucosamine hydrochloride	—	3	68·5
Glucosamine hydrochloride	—	24	73·0
N-Acetylglucosamine	—	3	27·0
N-Acetylglucosamine	—	24	42·0
A substance (preparation III)	—	3	0
A substance (preparation III)	—	24	0·075
A substance (preparation III)	Hydrolysed with N-acetic acid for 16 hr.	4	6·9
A substance (preparation III)	Hydrolysed with N-acetic acid for 16 hr.	24	7·9
A substance (preparation III)	Oxidized at pH 5 for 24 hr. and then hydrolysed with N-acetic acid for 16 hr.	24	19·5
A substance	Hydrolysed with 6 N-HCl for 2 hr.	3·5	47·3

Table 5. *Apparent fucose content of A substance and of some of its degradation products as determined by the acetaldehyde liberated on oxidation with periodate*

Substance	Treatment	Fucose (%)
Fucose	—	88, 92
A substance	—	0·30
A substance (preparation III)	—	0
A substance (preparation III)	Oxidized for 24 hr. at pH 5 and then hydrolysed at 100° with N-acetic acid for 16 hr.	0·98
A substance (preparation III)	N-Acetic acid, 16 hr.	11·6
A substance (preparation III)	N-Acetic acid, 48 hr.	12·3
A substance	N-Acetic acid, 48 hr.	12·9
A substance	N-Acetic acid, 16 hr. indiffusible material	0·56*

* In terms of the original A substance this represents 0·35% fucose.

maximum amino-acid N which is 38% of the total N of the A substance as determined by the ninhydrin method (Van Slyke, Dillon, MacFadyen & Hamilton, 1941).

Determination and characterization of the acetaldehyde formed on oxidation. Fucose and threonine give rise to acetaldehyde on oxidation with periodate under the same conditions (Nicolet & Shinn, 1941*a*; Shinn & Nicolet, 1941). The successful determination of threonine in Conway units (Winnick, 1942) suggested the adoption of the method for the determination of fucose and, although no serious attempt was made to develop an absolutely quantitative technique, yields of acetaldehyde equivalent to about 90% of the fucose taken were readily obtained.

$NaHSO_3$ (1·5 ml. of 0·25 M solution) was pipetted into the central chamber of a Conway unit. The substance to be analysed contained in a volume of 0·5 ml. was placed in the outer chamber and 1 ml. of 0·1 M-K_3PO_4 added. The concentration of K_3PO_4 was adjusted so that 1 ml. exactly neutralized, to pH 7·0, the 0·5 ml. of 0·2 M-HIO_4 which was finally added. The greased lid was then slipped into position, the unit was rotated in the usual manner, and kept at room temperature for the period of oxidation (2–5 hr., depending on temperature). A drop of 1% starch solution was then added to the inner chamber and the excess bisulphite oxidized with I_2 until a faint-blue tint was obtained. The aldehyde-bisulphite compound was decomposed with powdered Na_2HPO_4 or $NaHCO_3$ and titrated with 0·005 N-I_2. The results obtained with two preparations of A substance and some of their degradation products are given in Table 5. Fucose, included with each set of determinations, gives rather variable results; the average recovery of acetaldehyde was 90% of the theoretical. The values for the fucose content of the hydrolysis products are, therefore, corrected for a low recovery of this order. Threonine behaves more consistently and gives 95% of the theoretical yield of acetaldehyde. A substance does not liberate any appreciable amount of acetaldehyde during oxidation, hence acetaldehyde is not involved in the formaldehyde-dimedone determinations. The acetic acid hydrolysis products of A substance oxidized for 24 hr. at pH 5 give little acetaldehyde compared with the corresponding hydrolysate of the unoxidized A substance. The acetic acid hydrolysate of two preparations of A substance gave acetaldehyde equivalents to 12·3 and 12·9% fucose respectively. This is the maximum amount of free fucose obtained after mild acid hydrolysis and will be designated as acid-labile fucose. It represents about three-quarters of the total fucose, 18·5%, present in the A substance (Table 1). The indiffusible fraction of the A substance after a partial hydrolysis (16 hr., N-acetic acid) (Aminoff *et al.* 1948) has a total fucose content of 12·4% which, expressed in terms of the original A substance before hydrolysis, represents a fucose content of 7·7% (Table 1) and therefore still contains 2·2% of the original A substance as acid-labile fucose residues. This material yields a negligible amount of

acetaldehyde on oxidation with periodate, equivalent to a fucose content of 0·56% (i.e. 0·35% of the original A substance used for the hydrolysis).

The acetaldehyde obtained on the oxidation of the products of mild acid hydrolysis of A substance did not arise from threonine since chromatographic analysis shows that this amino-acid is not present in the free state in the acetic acid hydrolysates. Furthermore, the oxidation of the A substance with periodate involves all the fucose residues but not threonine, and, although an acetic acid hydrolysate of both the oxidized and the unoxidized A substance gave a little NH_3 on subsequent oxidation (Table 4), the former material gave very little acetaldehyde. The amount of acetaldehyde arising from threonine would, therefore, appear to be negligible in the mild acid hydrolysis products examined.

The acetaldehyde formed during the oxidation was characterized as the dimedone derivative by absorbing the volatile vapours produced in the outer chamber of the Conway unit into a solution of dimedone at pH 4·5 (acetate buffer), contained in the inner chamber. At the end of 24 hr. several large needle-shaped crystals were formed. After recrystallization from ethanol, the crystals melted sharply at 138–139° (cf. Weinberger, 1931). A mixture of the crystals obtained from the oxidation products of fucose and from an acetic acid hydrolysate of A substance showed no depression of melting point.

DISCUSSION

Course of oxidation. The rate of serological inactivation of A substance during oxidation with periodate, at pH values between 5 and 10, was most rapid at pH 5 (acetate buffer) and decreased with rise in pH, with no inactivation after 3 hr. at pH 10 (borate buffer). The stability at the higher pH is remarkable in view of the known lability of the blood-group substances to alkali. Although the presence of borate has little effect on the rate of reduction of periodate at pH 2·4 and 7·5 the slower rate of inactivation of the A substance at the higher pH values might well be due to the formation of chelation complexes with the *cis*-hydroxyl groups. Both serological properties are lost on oxidation, but the haemolysis-inhibiting (Forssman) activity is destroyed less rapidly than the capacity to inhibit iso-agglutination.

The pH values chosen to follow the course of oxidation were those optimal for the determination of the acid and aldehyde produced, namely pH 5·0 and 7·5 respectively. Although the rate of serological inactivation is less rapid at the higher pH, the oxidation is more extensive. Oxidation at pH 5 is essentially complete in about 6 hr., while after 3 days at pH 7·5 substantial reduction of the periodate is still occurring. A further marked difference in behaviour of the A substance is observed after the initial oxidation at pH 5 or 7·5. Thus re-oxidation of material previously oxidized at pH 5 results in further reduction of periodate only at pH 7·5, whereas the material initially oxidized at pH 7·5 reduces more periodate at both pH 5 and 7·5. This suggests that the initial oxidation at pH 7·5 exposes additional structures susceptible to oxidation. A similar instance of more extensive oxidation in an alkaline medium has been observed by Head (1947) during the oxidation of β-methylglucoside and β-methylcellobioside. The cause of this overoxidation was attributed by this author to the sensitivity of the primary oxidation products to hydrolysis by alkali with the production of structures susceptible to further oxidation. This interpretation is not applicable to our observations, since treatment of the material oxidized at pH 5 with sodium bicarbonate at pH 9·4 for 24 hr. and subsequent retreatment with periodate at pH 5 and 7·5 shows no difference in the behaviour from that of material oxidized at pH 5 without subsequent treatment with sodium bicarbonate. It must be concluded, therefore, that acid and alkaline periodate differ markedly in their oxidizing capacity.

Oxidation of the A substance reaches completion in an acid medium, but the amount of periodate reduced depends on the conditions used. Thus, in acetate buffer (pH 5) 1 g.mol. of periodate is reduced by 430 g. of A substance after 4 hr. and 364 g. after 24 hr., whereas the corresponding figure, the amount of A substance reducing 1 g.mol. of periodate, obtained when determining the formic acid produced on oxidation with sodium periodate at pH 4·5 is 260 g. after 26 hr. These values are a measure of the total reduction of periodate due to (*a*) specific oxidation of the sugar molecules containing adjacent, unsubstituted hydroxyl groups and (*b*) the nonspecific 'overoxidation' of the primary oxidation products.

Constituents involved in the oxidation. Removal, by dialysis, of excess periodate and buffer after oxidation and subsequent qualitative chromatographic analysis of the hydrolysates of the oxidation products reveals no change in the amino-acid composition of the A substance, but indicates the absence of galactose and fucose. Quantitative chromatographic analysis of the undegraded A substance shows the presence of glucosamine and chondrosamine in equimolecular proportions, whereas the corresponding hydrolysate of the oxidized material contains about three-quarters of the hexosamine originally present in the A substance and the ratio of glucosamine to chondrosamine is 2:1. Thus oxidation with periodate at pH 5 involves the fucose, galactose and half the *N*-acetylchondrosamine residues.

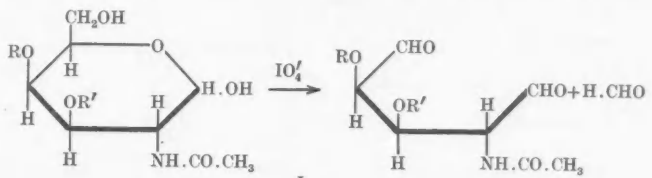
Simple substances liberated during oxidation. Formaldehyde and formic acid, but no acetaldehyde or ammonia, are produced during the oxidation of the A substance and the determination of the former pair of substances is not subject to interference by the latter.

1 g.mol. of formaldehyde is produced by the oxidation of 1964 g. of A substance. The formaldehyde

could arise from the serine, galactose or *N*-acetylhexosamine residues. No ammonia is liberated during the oxidation and hence serine is not involved.

The measurement of the acid produced presents no difficulty, the problem, however, devolves on the interpretation of the results obtained. Halsall *et al.* (1947) showed that the time required for the production of the theoretical amount of acid varied for different sugars and with both potassium periodate (Halsall *et al.* 1947) and sodium periodate (Potter & Hassid, 1948) the period of oxidation of a large molecular substance was empirically fixed by the time required to produce the theoretical amount of acid from a disaccharide which possessed the structural unit responsible for the acid production in the material under investigation. Thus β-methyl maltoside and maltose were chosen as the standard substances when determining the chain length of the glycogens and starches. The acid formed during the oxidation of the A substance could arise from the galactose, fucose or the reducing terminal *N*-acetylhexosamine residues, but in the absence of suitable simple derivatives of the latter two sugars, lactose and maltose, were used as standard substances to establish a suitable period for oxidation. Maclay, Hann & Hudson (1939) found that α- and β-methyl-L-fucosides reduced two molecules of periodate within 1 hr., but the acid formed was not determined. Lactose and maltose on oxidation with sodium periodate produce the expected quantity of formic acid after 25 hr. and about 2000 g. of A substance produce 1 g.equiv. of an acid after oxidation with either potassium or sodium periodate for the same length of time. More prolonged oxidation of lactose, maltose and the A substance by either method gives an increase in acid production.

The reducing end group. The blood-group A substance readily gives a colour with Ehrlich's reagent, under the conditions used for the determination of *N*-acetylglucosamine (Morgan & Elson, 1934) and the intensity of the colour is equivalent to approximately a quarter of the total calculated *N*-acetylhexosamine content (Aminoff *et al.* 1950). *N*-acetyl- and *N*-benzoyl-methylglucosaminides, on the other hand, do not give this reaction, and one might therefore conclude that the reactive *N*-acetylhexosamine residues in the A substance form the reducing end groups (I, where R or R′ is a glycoside or a peptide residue).



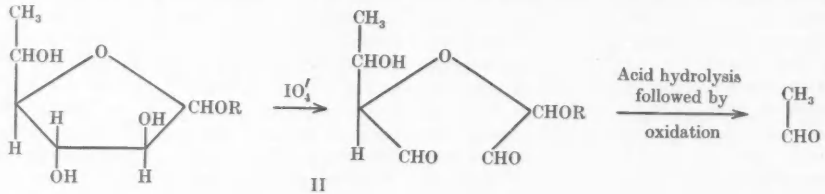
I

The yields of formaldehyde obtained from our purest material and from a partially purified preparation of A substance (preparation III) are proportional to the *N*-acetylhexosamine colour given by these materials and the origin of the formaldehyde could therefore be attributed to the reducing *N*-acetylhexosamine residues which on oxidation with periodate would result in cleavage of the C-5—C-6 linkage (I). This conclusion does not conflict with the observation that the A substance after oxidation develops the same amount of colour as does the original A substance when treated with mild alkali and Ehrlich's reagent, since the colour reaction only involves the groups at C atoms 1 and 2 of the *N*-acetylhexosamine molecule. That an *N*-acetylhexosamine residue is involved in oxidation is further substantiated by the observation that the hydrolysis products of the oxidized A substance show a decrease in hexosamine content, the value of which is again equivalent to a quarter of the total hexosamine. On the basis of quantitative paper-chromatographic analysis of the A substance oxidized at pH 5·0 and 7·5 the decrease in hexosamine content is found to be due principally to a loss in chondrosamine which would suggest that the terminal *N*-acetylhexosamine residue involved in the first stages of oxidation is *N*-acetylchondrosamine. It has been found, however, that *N*-acetylchondrosamine gives considerably less colour than *N*-acetylglucosamine and that the earlier report (Morgan & Elson, 1934), that equal weights of the two *N*-acetylhexosamines give the same amount of colour, is incorrect. This observation will be considered in greater detail in a further paper. As *N*-acetylchondrosamine gives about one-fifth of the colour given by *N*-acetylglucosamine one must conclude that *N*-acetylglucosamine also contributes to the total colour given by the A substance. The contribution of *N*-acetylglucosamine to the total colour can arise in two ways. First, by an *N*-acetylglucosamine residue also being present as a reducing end group. In that case the hydroxyl group on C atom 6 of the proposed *N*-acetylglucosamine end groups must be substituted to account for the low yield of formaldehyde which is equivalent to only one *N*-acetylhexosamine unit present as a reducing end group. A second possibility, as formulated in an earlier contribution (Aminoff & Morgan, 1949), is that the terminal *N*-acetylchondrosamine residue is linked

through C atom 1 of an *N*-acetylglucosamine residue, and that the mild alkaline treatment, under the conditions used for the determination of *N*-acetylhexosamine, breaks this glycoside linkage and thus transforms the *N*-acetylglucosamine into an end group and renders it reactive. These changes would satisfactorily account for the slower development of the maximum colour given by the A substance on heating with the alkali (14 min.) as compared with the time required (4 min.) for the free *N*-acetylhexosamines. Further evidence for differentiating between these two structural possibilities is now being sought.

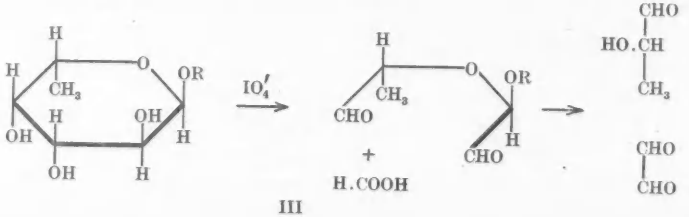
The mode of combination of fucose residues. The evidence for the form and sequence of fucose residues in the A substance is deduced from the following observations: (1) The total fucose content of the A substance is 18·5%. (2) Undegraded A substance gives no acetaldehyde on oxidation with periodate. oxidation at pH 5 for 24 hr., gives a negligible amount of acetaldehyde on re-oxidation with periodate. (8) The fucose content is little changed after oxidation of the A substance with sodium hypoiodite.

From the observations given under (2) and (8) above it is most probable that the fucose residues are not present as reducing end groups but form either non-reducing terminal residues or are situated in the main carbohydrate chain. Since three-quarters of the total fucose in the A substance is readily liberated on complete mild acid hydrolysis (48 hr. N-acetic acid), in an uncombined state and without extensive hydrolysis of the other carbohydrate constituents, it would appear that these fucose residues are terminal and are therefore present either as non-reducing end groups or are closely linked at the end of the chain. It might be concluded from the ease of hydrolysis of the glycoside bonds that the terminal fucose units possess a furanose structure (II). The



II

(3) Most of the fucose is readily liberated in an uncombined state by mild acid hydrolysis, i.e. N-acetic acid. (4) The maximum amount of acid-labile fucose is obtained by hydrolysis with N-acetic acid at 100° for 48 hr., as determined by the acetaldehyde liberated on oxidation with periodate, and represents 13% of the A substance (Aminoff *et al.* 1950), i.e. three-quarters of the total fucose. The remaining 5·5% of the fucose in the A substance represents the acid-stable fucose residues. (5) The indiffusible material obtained by dialysis of an incomplete (16 hr.) acetic acid hydrolysate of the A substance (Aminoff *et al.* 1948), a material which still contains some acid-labile fucose, gives no acetaldehyde on oxidation with periodate. (6) Oxidation of the A substance with periodate at pH 5 results in the destruction of all the fucose, i.e. both the acid-labile and acid-stable fucose residues. (7) The mild acid hydrolysis products of the A substance after oxidation of the A substance for 24 hr. at pH 5·0 with periodate would then presumably involve the hydroxyl groups at C atoms 2 and 3 and mild acid hydrolysis of the oxidation products will yield a material which on further oxidation should give rise to the same amount of acetaldehyde as is obtained from the undegraded A substance after acid hydrolysis and oxidation alone, viz. an amount equivalent to 13% of fucose. It has been found, however, that A substance after oxidation at pH 5 for 24 hr., followed by hydrolysis with acetic acid, and re-oxidation with periodate yields acetaldehyde equivalent to a content of less than 1% fucose. The results obtained, therefore, indicate that the hydroxyl groups at C atoms 3 and 4, at least, of the acid-labile fucose units, are involved in the initial oxidation and must therefore be unsubstituted. The acid-labile fucose units, in spite of their acid lability, would therefore appear to be present as pyranosides.



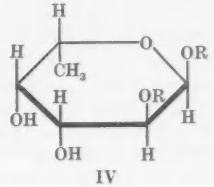
III

The acid produced during the oxidation of the A substance with periodate can therefore be attributed to the terminal fucose residues, with the hydroxyl groups on C atoms 2, 3 and 4 unsubstituted, as shown in III. The amount of acid produced on oxidation of the A substance for 25 hr., however, corresponds to only two-thirds of the acid-labile fucose, and therefore to a half of the total fucose content of the A substance.

Since no acetaldehyde is formed on oxidation of the mild acid hydrolysis products of the A substance oxidized at pH 5 (Table 5) the hydroxyl groups at C atoms 3 and 4 of all the acid-labile fucose residues are involved in the initial oxidation. The non-terminal acid labile fucose residues (IV), representing a quarter of the total fucose, are presumably substituted at C atoms 1 and 2 in the undegraded A substance. (R is a glycoside or peptide residue.)

Assuming, as seems most probable, that the terminal and non-terminal acid-labile fucose residues are closely linked, two modes of combination are possible (V or VI).

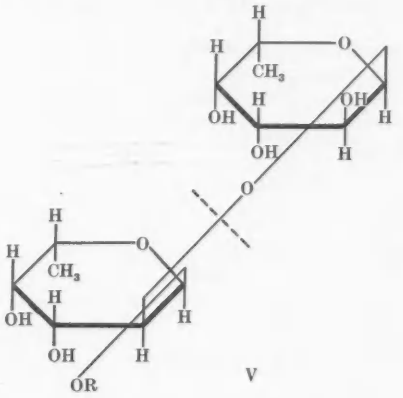
After hydrolysis of A substance for 16 hr. with acetic acid (Aminoff *et al.* 1948) 38 % by weight diffuses through a cellophan membrane. The indiffusible residue contains all the acid-stable fucose (about a quarter of the total fucose originally present in the A substance) together with about 14 % of the acid-labile fucose (three-quarters of the total fucose) which has not been liberated owing to the hydrolysis being incomplete after 16 hr. No significant amount of acetaldehyde was obtained on oxidation of this



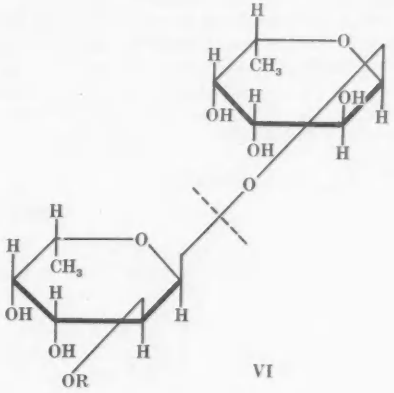
IV

indiffusible material (Table 5) and thus C atom 1 of the non-terminal acid-labile fucose residue in this partial acid hydrolysis product is still glycosidically bound, and the two fucose residues are therefore presumably linked as shown in V. The configuration of the glycoside linkages is not known.

The acid-stable fucose residues, which represent the remaining quarter of the total fucose of the A substance, are also, like the acid-labile fucose residues, involved in the oxidation with periodate as



V



VI

Fucose^1—^{2}Fucose^1—R

↓

Fucose + Fucose^1—R

↓

No acetaldehyde

Partial mild hydrolysis with acetic acid

Oxidation of indiffusible moiety

Fucose^1—^{1}Fucose^2—R

↓

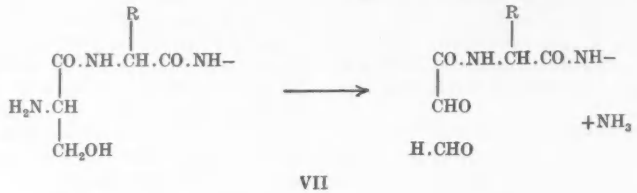
Fucose + Fucose^2—R

↓

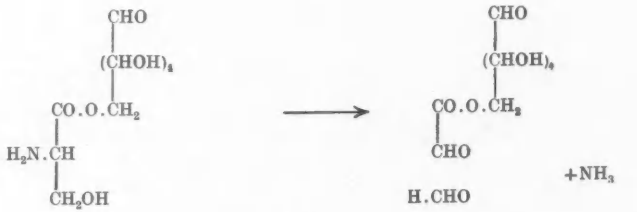
Acetaldehyde

demonstrated by the negligible fucose content of the A substance after oxidation at pH 5·0 for 24 hr. (Table 1). The hydroxyl group on C atom 3 of the acid-stable fucose residues must therefore be unsubstituted. The other unsubstituted hydroxyl group is most probably on C atom 2, in contrast to the acid-labile non-terminal fucose residue (IV) which has a substituted hydroxyl group at C atom 2. As such this acid-stable fucose residue would be apparently less sensitive to acid hydrolysis since both the linkages at C atoms 1 and 4 must be broken before acetaldehyde is liberated on oxidation with periodate; whereas the liberation of acetaldehyde from a fucose residue linked at C atoms 1 and 2 (IV) depends on the fission of only the linkage involving C atom 1.

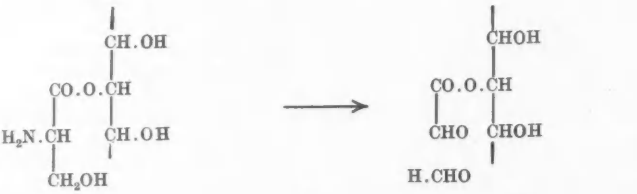
reducing terminal residues. The evidence for the mode of combination of the galactose and *N*-acetylglucosamine residues in the A substance is at present only circumstantial and is derived from the study of the mild acid hydrolysis products of this material. The undegraded A substance shows no pneumococcal type XIV or anthrax C polysaccharide specificity, but both these immunological characters, and an enhancement in the pre-existing heterophile (or Forssman) property, develop as a result of mild hydrolysis with acid (Ivanovics, 1940*b*; Kabat, Baer, Bezer & Knaub, 1948; Aminoff *et al.* 1948). It is well known that the immunologically active polysaccharides obtained from the Forssman antigen, the pneumococcal type XIV and the anthrax C antigens contain *N*-acetylglucosamine and a hexose which is



VII



VIII



IX

The galactose and N-acetylglucosamine residues. The production of formaldehyde, formic acid and acetaldehyde under different conditions has given some indication of the position of linkages joining the various fucose residues, and also the *N*-acetylchondrosamine end group in the A substance. The yield of formaldehyde and formic acid is used to determine the chain length; the formaldehyde liberated is a measure of the number of reducing end groups and the formic acid of the number of non-reducing terminal residues. The evidence for the mode of combination of the galactose and *N*-acetylglucosamine residues in the A substance is at present only circumstantial and is derived from the study of the mild acid hydrolysis products of this material. The undegraded A substance shows no pneumococcal type XIV or anthrax C polysaccharide specificity, but both these immunological characters, and an enhancement in the pre-existing heterophile (or Forssman) property, develop as a result of mild hydrolysis with acid (Ivanovics, 1940*b*; Kabat, Baer, Bezer & Knaub, 1948; Aminoff *et al.* 1948). It is well known that the immunologically active polysaccharides obtained from the Forssman antigen, the pneumococcal type XIV and the anthrax C antigens contain *N*-acetylglucosamine and a hexose which is most probably galactose (Brunius, 1936; Chase & Landsteiner, 1939; Goebel, Beeson & Hoagland, 1939; Ivanovics, 1940*a*). It seems probable, therefore, that the *N*-acetylglucosamine and galactose units are closely associated in the A substance and in its mild acid hydrolysis products, and account for the cross-reactivity observed.

The yield of formaldehyde on oxidation of the A substance is fully accounted for by the reducing *N*-acetylchondrosamine residue, and hence the oxida-

tion of galactose presumably involves rupture between C atoms 2 and 3 or possibly 3 and 4.

The amino-acid moiety. According to Nicolet & Shinn (1939) and Posternak & Pollaczek (1941) the acetylated hydroxyamino-acids give no ammonia when treated with periodate. *N*-Acetylglucosamine, however, gives an appreciable amount of ammonia. Neuberger (1941) also detected ammonia in the oxidation products of ethyl *N*-benzoylglucosamate. In these instances, therefore, the acyl group offers little or no protection to the amino group. Undegraded A substance gives no ammonia and hence the hydroxyl groups attached to carbon atom 3 in the *N*-acetylhexosamine residues are most probably substituted. In spite of the high content of the hydroxyamino-acids in the amino-acid moiety of the A substance (34% of the maximum amino-acid nitrogen is present in the form of hydroxyamino N), neither serine nor threonine can be situated at the end of a peptide chain (VII) or be attached by ester linkages involving the carboxyl group to the primary (VIII) or secondary (IX) alcohol groups in the sugar residues.

Certain of the hydroxyl groups of fucose, glactose and part of the hexosamine units are unsubstituted, to permit oxidation, and thus the points of attachment of the amino-acid residues are limited to the hydroxyl groups that have not been involved in oxidation.

Conclusions. On the basis of the analytical results available at present, the carbohydrate constituents of the A substance, galactose, fucose, *N*-acetylglucosamine and *N*-acetylchondrosamine, are present in equimolecular proportions and correspond to a unit with a minimum molecular weight of 968 (Aminoff *et al.* 1950). The oxidation results, however, suggest that the smallest unit with a reducing end group and a non-reducing terminal residue and containing three different forms of fucose is about 3880 and would thus contain four galactose, four fucose, four *N*-acetylglucosamine and four *N*-acetylchondrosamine residues. This represents an 'average' and not necessarily a repeating structural unit.

The results of periodate oxidation taken in conjunction with those of mild acid and alkaline hydrolysis have suggested the form and sequence of certain of the sugar molecules in the carbohydrate moiety of the A substance. Further investigations to establish the structure of this important biological material are in progress.

SUMMARY

1. Fucose, galactose and a half of the total *N*-acetylchondrosamine residues of the A substance are oxidized by periodate at pH 5.

2. Formaldehyde and formic acid, but no acetaldehyde or ammonia, are liberated during the oxidation.

3. The formaldehyde and formic acid (25 hr. oxidation) are produced in approximately equimolecular proportions and correspond to a minimum molecular weight of the order of 2000.

4. The hydroxyamino-acids, serine and threonine, are not present as terminal residues in the amino-acid-containing moiety and are not involved as single molecules in simple esterification with the hydroxyl groups of the sugar units.

5. Certain aspects of the structure of the A substance are discussed in the light of the results obtained.

REFERENCES

Aminoff, D. & Morgan, W. T. J. (1948). *Nature, Lond.*, **162**, 579.

Aminoff, D. & Morgan, W. T. J. (1949). *Biochem. J.* **44**, xxi.

Aminoff, D., Morgan, W. T. J. & Watkins, W. M. (1948). *Biochem. J.* **43**, xxxvi.

Aminoff, D., Morgan, W. T. J. & Watkins, W. M. (1950). *Biochem. J.* **46**, 426.

Baer, H., Dische, Z. & Kabat, E. A. (1948). *J. exp. Med.* **88**, 59.

Bell, D. J. (1948). *J. chem. Soc.* p. 992.

Bendich, A., Kabat, E. A. & Bezer, A. E. (1947). *J. Amer. chem. Soc.* **69**, 2163.

Bray, H. G., Henry, H. & Stacey, M. (1946). *Biochem. J.* **40**, 124.

Brunius, F. E. (1936). *Chemical Studies on the True Forssman Hapten, the corresponding Antibody, and Their Interaction.* Stockholm: Fahlcrantz.

Chase, M. W. & Landsteiner, K. (1939). *Ann. Rev. Biochem.* **8**, 579.

Dische, Z. & Shettles, L. B. (1948). *J. biol. Chem.* **175**, 595.

Dische, Z., Shettles, L. B. & Osnos, M. (1949). *Arch. Biochem.* **22**, 169.

Elson, L. A. & Morgan, W. T. J. (1933). *Biochem. J.* **27**, 1824.

Goebel, W. F., Beeson, P. B. & Hoagland, C. L. (1939). *J. biol. Chem.* **129**, 455.

Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. (1943). *Biochem. J.* **37**, 538.

Halsall, T. G., Hirst, E. L. & Jones, J. K. N. (1947). *J. chem. Soc.* pp. 1399, 1427.

Head, F. S. H. (1947). *J. Text. Inst., Manchr*, **38**, T. 389.

Ivanovics, G. (1940*a*). *Z. ImmunForsch.* **97**, 402.

Ivanovics, G. (1940*b*). *Z. ImmunForsch.* **98**, 373.

Jackson, E. L. (1944). *Organic Reactions*, vol. II, p. 341. New York: J. Wiley.

Jones, J. K. N. (1946). *Rep. Progr. Chem.* **43**, 180.

Kabat, E. A., Baer, H., Bezer, A. E. & Knaub, V. (1948). *J. exp. Med.* **88**, 43.

Kabat, E. A., Bendich, A., Bezer, A. E. & Knaub, V. (1948). *J. exp. Med.* **87**, 295.

Ma, T. S. & Zuazaga, G. (1942). *Industr. Engng Chem.* (Anal. ed.), **14**, 280.

Maclay, W. D., Hann, R. M. & Hudson, C. S. (1939). *J. Amer. chem. Soc.* **61**, 1660.

Morgan, W. T. J. (1946). *Biochem. J.* **40**, xv.

Morgan, W. T. J. & Elson, L. A. (1934). *Biochem. J.* **28**, 988.
Morgan, W. T. J. & King, H. K. (1943). *Biochem. J.* **37**, 640.
Neuberger, A. (1941). *J. chem. Soc.* p. 47.
Nicolet, B. H. & Shinn, L. A. (1939). *J. Amer. chem. Soc.* **61**, 1615.
Nicolet, B. H. & Shinn, L. A. (1941*a*). *J. Amer. chem. Soc.* **63**, 1456.
Nicolet, B. H. & Shinn, L. A. (1941*b*). *J. biol. Chem.* **139**, 687.
Owen, L. N. (1943). *Rep. Progr. Chem.* **40**, 115.
Partridge, S. M. (1948*a*). *Biochem. J.* **42**, 238.
Partridge, S. M. (1948*b*). *Biochem. J.* **42**, 251.
Posternak, T. & Pollaczek, H. (1941). *Helv. chim. Acta*, **24**, 1190.
Potter, A. L. & Hassid, W. Z. (1948). *J. Amer. chem. Soc.* **70**, 3488.
Rees, M. W. (1946). *Biochem. J.* **40**, 632.
Reeves, R. E. (1941). *J. Amer. chem. Soc.* **63**, 1476.
Shinn, L. A. & Nicolet, B. H. (1941). *J. biol. Chem.* **138**, 91.
Van Slyke, D. D., Dillon, R. T., Macfadyen, D. A. & Hamilton, P. (1941). *J. biol. Chem.* **141**, 627.
Weinberger, W. (1931). *Industr. Engng Chem.* (Anal. ed.), **3**, 365.
Winnick, T. (1942). *J. biol. Chem.* **142**, 461.

Kinetic Studies of the Metabolism of Foreign Organic Compounds

1. THE FORMATION OF BENZOIC ACID FROM BENZAMIDE, TOLUENE, BENZYL ALCOHOL AND BENZALDEHYDE AND ITS CONJUGATION WITH GLYCINE AND GLUCURONIC ACID IN THE RABBIT

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Investigations carried out in this laboratory in recent years (summarized by Thorpe, 1950) have dealt with the factors determining the metabolic pathways followed by foreign aromatic compounds when several such pathways are available. While it is to be expected that all possible reactions will occur simultaneously, the proportions of the dose which actually follow the various routes differ greatly. Many factors may theoretically contribute to these differences, e.g. the efficiency of the necessary enzyme systems, the availability of conjugating molecules (e.g. glycine) and the availability of free energy for synthetic reactions. All such factors, taken together, determine the velocity of each individual reaction in the intact animal, so that knowledge of the relevant velocity constants is likely to prove of value in considering the detailed metabolism of foreign organic compounds. Since such information does not appear to be available in the literature we have planned a series of investigations in which we hope to compare the velocities of typical metabolic reactions which foreign organic compounds may undergo in the rabbit. In this first investigation the formation of benzoic acid from various precursors and its conjugation with glycine and glucuronic acid have been studied. The method consisted essentially of analysis of the individual urine samples passed by rabbits which had received the compounds under investigation. The metabolites estimated were ether-soluble acid (in some experiments fractionated into benzoic and hippuric acids) and ester glucuronide.

When a compound is administered to a rabbit it may undergo several consecutive processes, e.g. absorption, conversion of potential centre (see Bray, Ryman & Thorpe, 1948), conjugation and excretion. Preliminary experiments showed that absorption appeared to be virtually complete within about 2·5 hr., so that if measurements were made only on those portions of the excretion curves corresponding to times after this, the effect of absorption could be neglected. For the rest it was assumed that the curves obtained would represent the slowest process of the series. Thus if the amount of the compound administered is the only limiting factor then it would be expected that the reaction being studied would be of the first order.

METHODS

Animals, diet and dosage. Rabbits of not more than 2 kg. weight were used, since larger animals were liable to pass urine samples greater in volume than the capacity of the receivers used (140 ml.). They were maintained on the constant diet of rabbit pellets and water customary in this laboratory (Bray, Ryman & Thorpe, 1947). The compounds were administered by stomach tube as solutions or suspensions in water.

Collection of urine samples. For this purpose a fraction collector was constructed. It consisted of a brass turntable which carried round its periphery forty-eight receiving tubes each of capacity 140 ml. Its movement was weight-activated and controlled by an escapement operated by an electromagnet activated by impulses from a timing clock. In this way the receiver could be changed automatically at predetermined time intervals ranging from 1 min. to 1 hr. The rabbit was housed in a metabolism cage fitted into a tinned-copper collecting funnel of the usual type, the spout of which was connected to a glass tube delivering into the receivers beneath.

Estimation of metabolites. Ether-soluble acid was determined as previously described (Bray, Neale & Thorpe, 1946), fractionation being achieved by extraction of ether-soluble material with light petroleum (b.p. 40–60°) using three successive volumes of 25 ml. The method was adapted for use on blood samples (2 ml.). These were collected from a marginal ear vein directly into H_2SO_4 (15 ml., 0·083 N) and subsequently made up to 25 ml. with acid of the same strength. A sample of this (24 ml.) was transferred to an extractor and the protein precipitated by means of sodium tungstate (3 ml., 10% w/v). The resulting mixture was allowed to stand for 5 min., further acidified by the addition of 2 N-H_2SO_4 (5 ml.) and continuously extracted with ether for 6 hr. The ether-soluble acid was titrated with NaOH (0·01 N). Fractionation was carried out as for urinary acid. Control experiments showed that benzoic and hippuric acids added to normal rabbit blood (100–500 mg./100 ml. blood) could be recovered by this method to extents of 101 and 105%, respectively (ranges 96–109% and 100–109%, respectively). Ester glucuronide in urine was estimated by the reducing method previously described (Bray *et al.* 1946).

Plan of experiments. The overall plan was similar to that used in most metabolic investigations on the intact animal. A 'baseline' was determined during the day before the main experiment. Since the ingestion of food was followed by a sudden increase in the baseline value the experiments were carried out on fasting animals, beginning at approx. 10.30 a.m. The food ration was given at approx. 7 p.m. when most of the administered compound had been excreted. The importance of a constant food ration, adequate yet small enough to be ingested almost immediately, is obvious. Rabbits normally urinate only once or twice during the day time, and in order to obtain a sufficient number of urine samples during the experiment water (100 ml.) was given three or four times in the course of the day. Occasionally a rabbit passes a small urine sample when the bladder is full, shortly before the main sample. This leads to an error in the corresponding point, but since the values plotted are cumulative this is corrected in the next point (e.g. see Figs. 1 and 3, points at 5 hr. 20 min.). Two or three blood samples were withdrawn during the experiment. It will be seen from Table 1 that in some experiments glycine was given with the compound under investigation. Griffith & Lewis (1923) found that the average rate of excretion of conjugated benzoic acid by the rabbit on a normal diet was 68·7 mg./hr./kg. and after administration of glycine (3 equiv.) 111·7 mg./hr./kg. Consequently, we administered glycine (usually 3–5 equiv.) with the precursor in experiments in which the dose was relatively large to obtain rapid formation and excretion of hippuric acid and to prevent the accumulation of benzoic acid in the blood.

RESULTS

The treatment of the experimental results is explained by means of the protocols and calculation of the results of two typical experiments. The experiments can be divided into two groups: (1) the formation and conjugation of benzoic acid formed from administered toluene, benzyl alcohol, benzaldehyde and benzamide, and (2) the conjugation of benzoic acid administered as such. It should be noted that results have been expressed as benzoic acid throughout.

(1) *Exp.* 23, *rabbit no.* 301

Day 1. Baseline determined. Total excretion of ether-soluble acid during 23 hr., 399 mg. benzoic acid.

Day 2. Dose toluene (1·3 g.) + glycine (3 g.), at 11.10 hr. Water (100 ml.) given at 11.10, 12.30, 15.30 and 16.50 hr. Urine samples passed at 11.10, 14.15, 16.30, 16.50, 17.50, 19.45, 03.10 and 09.00 hr. Blood samples taken at 12.30 and 15.30 hr.

No increase was observed in the ether-soluble acid content of either sample of blood, and there was no increase in the ester glucuronide content of the urine throughout the experiment. The curves in Fig. 1

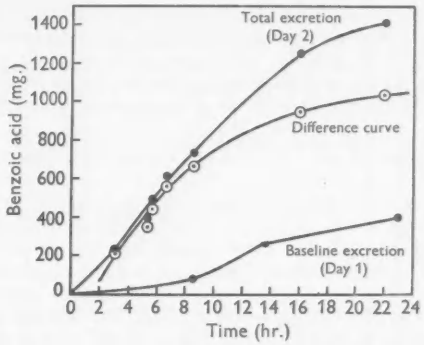

Fig. 1. Excretion of ether-soluble acid (expressed as benzoic acid) by rabbit no. 301 after administration of toluene (1·3 g.).

show the excretion of ether-soluble acid (expressed as benzoic acid) on days 1 and 2 and the 'difference curve', i.e. the baseline values obtained on day 1 have been subtracted from the results of day 2 and so represent the excretion of ether-soluble acid (expressed as benzoic acid), derived from the toluene administered.

Calculation of results

If the conversion of the precursor to benzoic acid is a first order reaction, then

$$db/dt = k_b a, \qquad (1)$$

where a and b are the amounts of precursor and benzoic acid in the body, and k_b the velocity constant for formation of benzoic acid. If the precursor undergoes other processes in addition to the formation of benzoic acid, and assuming a first-order mechanism for these, then

$$-da/dt = Ka, \qquad (2)$$

where K is the velocity constant for total excretion by all routes. Thus

$$k_b/K = E_b/E = E_{max.}/\text{dose}, \qquad (3)$$

where E_b is the amount of benzoic acid (and its conjugates) excreted in a given time, E the total amount

of precursor in all its forms excreted in the same time, and $E_{max.}$ the final amount of benzoic acid (and its conjugates) excreted. Therefore

$$k_b/K = (E_b - E_{max.})/(E - \text{dose}). \quad (4)$$

Let $$E_{max.} - E_b = a_b$$

(i.e. the amount of toluene in the body which is destined for oxidation to benzoic acid). Then from (4), $k_b = Ka_b/a$, since dose $- E = a$. Hence

$$db/dt = Ka_b. \quad (5)$$

Integration of equation (5) gives

$$K = (\ln a_{b_1} - \ln a_{b_2})/(t_2 - t_1).$$

The total excretion E_b (free and conjugated benzoic acid, expressed as benzoic acid) is plotted against time (t) to give the 'difference' curve as shown in Fig. 2. An approximate asymptote ($E_{max.}$), representing the total excretion of toluene as benzoic acid

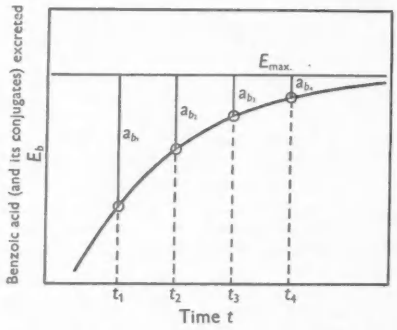

Fig. 2. Determination of a_b values from 'difference' curve (diagrammatic). ⊙ = Point determined by experiment.

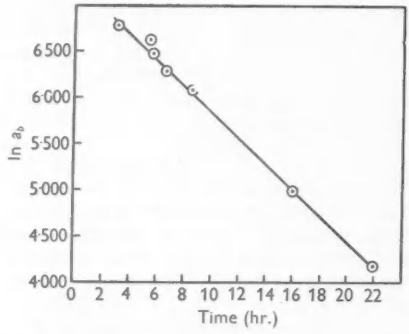

Fig. 3. Values of ln a_b for rabbit no. 301 plotted against t.

and its conjugates, is derived using the property of exponential curves whereby equal percentages of the amount of precursor remaining in the body are converted in equal periods of time. Values of a_b, i.e. ($E_{max.} - E_b$), corresponding to various times, t_1, t_2, t_3, etc., i.e. a_{b_1}, a_{b_2}, a_{b_3}, etc., are determined and ln a_b plotted against t as in Fig. 3. For a first-order reaction this graph should be linear, and by a process of successive approximations the value for $E_{max.}$ is determined which will in fact fulfil this condition. The slope of this line is $-K$. By then drawing the graph of E_b and t derived from K the fit of the experimentally determined points can be ascertained. In most experiments these conditions were fulfilled within reasonable experimental error. The following values were obtained by this treatment of the results of Exp. 23: $K = 0{\cdot}14$ hr.$^{-1}$; $E_{max.} = 1090$ mg. benzoic acid (= 63·5 % of dose) and

$$k_b = 0{\cdot}14 \times 63{\cdot}5/100 = 0{\cdot}09 \text{ hr.}^{-1}.$$

In the case of toluene a part of the dose is known to be eliminated via the lungs and approximately 60 % is converted to benzoic acid. Since the experimental results fit an exponential curve it follows that elimination of the other 40 % is according to a first-order reaction.

Application to other precursors of benzoic acid. If excretion unchanged takes place, as in the expiration of toluene, k_b may be calculated from K as in the above example, e.g. if 63·5 % of the dose is excreted as benzoic acid and its conjugates, then $k_b = 0{\cdot}635K$.

If modification of the potential centre for conjugation takes place before excretion in some form not estimated, then K may be taken as the velocity constant for the conversion of the potential centre (k_b). If the unknown processes are not of the first order, the error involved in the above assumption will be reflected in the excretion curves and the 'fit' with the theoretical curve will be correspondingly less close. It will be seen from Table 1 that approximately 70–90 % of doses of the compounds fed (with the exception of toluene) were in fact excreted as benzoic acid and its conjugates. Since the fate of the remainder is uncertain K is taken as k_b and any error incurred will be relatively small.

(2) *Exp.* 1, *rabbit no.* 309

Day 1. Baseline determined. Total excretion of ether-soluble acid in 5·7 hr. = 75 mg. (as benzoic acid). Total excretion of reducing material in 5·7 hr. = 17 mg. (as benzoic acid).

Day 2. Dose sodium benzoate (1·25 g.) at 09.00 hr. Water (100 ml.) at 09.00, 10.25, 12.20, 14.30 hr. Urine samples passed at 09.45, 10.25, 11.35, 11.55, 12.25, 12.50, 13.25, 14.05, 15.10, 16.10 hr. Blood samples taken at 11.35, 12.50, 15.10 hr.

The 'difference' excretion curves for ether-soluble acid and for glucuronide are shown in Figs. 4 and 5.

Blood analyses showed that during the experiment approximately 14 % of the free benzoic acid in the body was present in the blood, but no rise in the blood hippuric acid level was observed. (The blood

volume was taken as 6·7 % of the body weight, on the basis of four determinations on rabbits made for us by Dr M. E. Nutt and Miss D. M. Jackson by the dye (T-1824) method of Courtice & Gunton, 1949.)

The formation of hippuric acid took place at a constant rate (k_h), so that further mathematical treatment of these results is unnecessary. The formation of glucuronide can now be considered in detail.

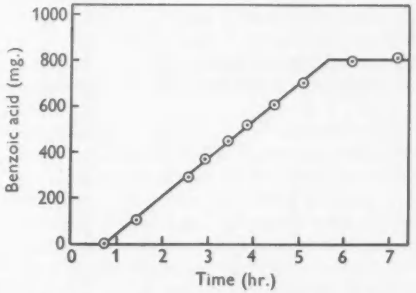

Fig. 4. 'Difference' excretion curve for ether-soluble acid (expressed as benzoic acid) for rabbit no. 309 after administration of sodium benzoate (1·25 g.).

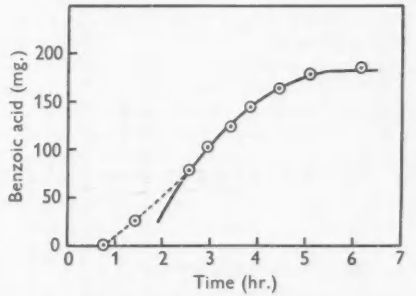

Fig. 5. 'Difference' excretion curve for glucuronide (expressed as benzoic acid) for rabbit no. 309 after administration of sodium benzoate (1·25 g.).

For a first-order reaction $dg/dt = k_g b$, where b is the total amount of benzoic acid present in the body, g the amount of glucuronide excreted and k_g the velocity constant for formation of benzoylglucuronide. The total excretion curve of E_b and t (E_b = ether-soluble acid and glucuronide) was plotted and also the glucuronide excretion curve of g and t.

Tangents to the second curve (dg/dt) were determined and plotted against b, which was determined from the first graph as the difference between the total excretion of benzoic acid ($E_{max.}$) and E_b. The slope of this line is k_g. The 'fit' of the curve (g, t) derived from k_g was checked with the experimental results. In Exp. 1 this mathematical treatment gave $k_g = 0{\cdot}11$ hr.$^{-1}$; $k_h = 168$ mg. hr.$^{-1}$.

Summary of results

Relevant results from all the experiments carried out are summarized in Table 1. It may be seen from the final column of Table 1 that straight-line excretion curves were sometimes obtained for hippuric acid after the administration of precursors as well as for benzoic acid itself. This happens when the initial rate of conversion of the precursor is greater than the maximum rate of glycine conjugation (see also Discussion, p. 94). Where these rates are approximately equal, small variations in k_h may be critical and either exponential curves or initially straight lines may be obtained. When the initial rate of conversion of the precursor exceeds the maximum rate of glycine conjugation benzoic acid would be expected to accumulate in the blood. This occurred in Exps. 17 and 18 with benzyl alcohol. In six experiments where benzoic acid was administered as sodium benzoate it was found that in fourteen determinations an average of 12 % (range 6–24 %) of the benzoic acid in the body was present in the blood. We made no attempt to determine the amount of glucuronide in the blood, owing to the practical difficulty of estimating small amounts in the presence of relatively large amounts of other reducing substances. Csonka (1924), however, found no increase in reducing material in the blood of the pig in 5 hr. following the administration of benzoic acid, although benzoylglucuronide was excreted during the first 6 hr. It may, therefore, be suggested that rapid excretion of benzoylglucuronide might also occur in the rabbit.

It has been shown that velocity constants of enzymic reactions increase with increasing temperature (see Wilson, 1949). We did not determine the body temperature of our experimental animals and it may, in fact, have varied, since Griffith (1938) observed that the administration of benzoic and hippuric acids to rats caused a fall in body temperature. It may be significant that vasoconstriction was observed in the ears of some animals after giving benzoic acid. While we found some variation in the individual values for k for each reaction, most variation occurred in the values found for benzoylglucuronide formation, which were much greater than could be accounted for by experimental error.

DISCUSSION

Conjugation and excretion of benzoic acid

Hippuric acid formation takes place at a constant rate and the acid is excreted immediately up to a limiting rate which appears to vary considerably between different rabbits. In two experiments (Exps. 4 and 5, Table 1), in which hippuric acid was found in the blood after feeding benzoic acid and glycine, values of k_h of 270 and 480 mg. hr.$^{-1}$ (as

Table 1. *Kinetics of formation and conjugation of benzoic acid in the rabbit*

Exp. no.	Rabbit no.	Dose (g.)*	Percentage of dose accounted for (*A*)	Percentage of *A* excreted as: Glucuronide	Ether-soluble acid	Hippuric acid	Benzoic acid	k_g (hr.$^{-1}$)	k_h (mg. hr.$^{-1}$)	k_b (hr.$^{-1}$)	Remarks (see footnote)
						Sodium benzoate					
1	309	1·25	95	19	81	78	3	0·11	168	—	*b, d*
2	309	1·25	92	16	84	83	1	0·09	163	—	*b, d*
3	263	0·84	81	14	86	86	0	0·05	115	—	*b, d*
4	301	3·0 (2)	108	12	88	87	1	0·08	480	—	*a, b, d*
5	302	2·5 (4)	72	—	100	—	—	—	270	—	*a, b, d, e, h*
6	299	1·25	76	—	100	—	—	—	142	—	*b, e, d, h*
							Average	**0·08**			
						Benzamide					
7	288	0·8	88	5	95	—	—	—	—	0·30	*b, f*^{5}*, g*
8	263	0·8	67	6	94	—	—	—	—	—	*b, e, f*^{7}
9	301	0·8 (5)	69	0	100	—	—	—	—	0·35	—
10	304	0·8 (5)	86	2	98	—	—	—	—	0·32	*f*^{2}
11	308	0·7 (6)	84	0	100	—	—	—	—	0·29	—
									Average	**0·32**	
						Benzaldehyde					
12	301	1·0	90	5	95	—	—	—	—	0·30	*b, f*^{6}
13	262	1·5 (3)	98	0	100	—	—	—	—	0·20	—
14	299	1·0 (4)	80	0	100	—	—	—	—	0·50	—
15	309	1·0 (4)	86	1	99	—	—	—	—	—	*e*
16	299	0·5 (8)	87	0	100	—	—	—	—	—	*e*
									Average	**0·33**	
						Benzyl alcohol					
17	306	1·0	82	24	76	—	—	—	—	0·80	*b, c, d*
18	306	1·6 (3)	79	9	91	—	—	—	—	0·90	*b, c, d*
19	301	0·5 (8)	52	3	97	—	—	—	—	1·00	*f*^{2}
20	302	0·5 (4)	84	2	98	—	1	—	—	1·00	*f*$^{2\cdot5}$
							Average of Exps. 19 and 20			**1·00**	
						Toluene					
21	279	1·3	45	0	100	—	—	—	—	0·16	—
22	299	1·0	63	0	100	—	—	—	—	0·11	—
23	301	1·3 (3)	64	0	100	—	—	—	—	0·09	—
24	301	1·3 (3)	58	0	100	—	1	—	—	0·09	—
25	303	2·6	40	4	96	—	—	—	—	—	*e, h*
									Average	**0·11**	

* Glycine administered (equivalents) shown in brackets.

a Hippuric acid was shown to be present in the blood during the experiment.
b Hippuric acid excretion curve was initially linear.
c The fact that the excretion curve was initially linear made it impossible to calculate k_b from the experimental results by the general method described in the text. An approximate value for k_b was calculated from the observed glucuronide values using equation (9) of the appendix.
d Benzoic acid was shown to be present in the blood during the experiment.
e Velocity constants were not calculated owing to insufficient data.
f^n Glucuronide was excreted during first n hr.
g Value for k_b calculated in usual way but only approximate since hippuric acid excretion curve initially linear.
h Experiment concluded before completion of excretion.

benzoic acid) were found, assuming that hippuric acid was present only in blood and urine. For the three rabbits used in Exps. 1–3 and 6, in which benzoic acid alone was given, the average rate of formation and excretion of hippuric acid was 147 mg./hr. This agrees with the work of Griffith & Lewis (1923) and Csonka (1924), which suggests that the availability of glycine controls the rate of hippuric acid formation. Griffith & Lewis found that the dose of glycine giving a maximum rate of excretion of hippuric acid was approximately 3 equiv., but it should be noted that no determinations have been made of the maximum rate of hippuric acid formation.

The results given in this paper show that the conjugation of benzoic acid with glucuronic acid follows the kinetics of a first-order reaction with a velocity constant of 0·08 hr.$^{-1}$. It will be seen from Table 1 that the percentage of a dose of benzoic acid excreted conjugated with glucuronic acid depends on the dose level and whether or not glycine is administered with the dose. In a previous paper (Bray, Humphris & Thorpe, 1949), it was shown by a comparison of results obtained with benzoic acid and benzamide, and with the toluic acids, toluamides and xylenes, that the highest percentages of glucuronide conjugation were observed when the parent acids were fed, presumably because they gave rise to higher blood levels than did the administration of precursors. Glycine conjugation appeared to be affected to a much smaller extent. It was suggested that at higher blood levels the glycine conjugation mechanism was overwhelmed and that glucuronide conjugation was in the nature of a 'shock mechanism'. It is clear, however, from the results of the present investigation, that this is only partially true, at least for benzoic acid and its precursors. The rate of hippuric acid formation is independent of the amount of benzoic acid in the body probably for amounts down to 25 mg./kg. (by analogy with man, from data of Quick, 1931). If the amount of benzoic acid present in the body is sufficiently small, the rate of glycine conjugation will be proportional to it and the kinetics of the conjugation would show a change to those of a first-order process. These conditions would be expected to prevail when the rate of conversion of a benzoic acid precursor is less than the maximum rate of glycine conjugation, i.e. when the excretion of hippuric acid follows an exponential curve (see later section on conversion of potential centres). Under these conditions no significant glucuronide excretion was observed, but, making allowance for analytical error, as much as 3 % of the amount of the dose excreted may have been present as glucuronide. Thus a minimum value for the velocity constant for hippuric acid may be deduced, as follows: The ratio of glycine conjugation to glucuronide conjugation is 97 : 3; therefore, since $k_g = 0{\cdot}08$, the velocity constant for hippuric acid formation must be greater than $0{\cdot}08 \times 97/3 = 2{\cdot}6$. Glucuronic acid conjugation is proportional to the amount of benzoic acid in the body. Thus an increase in the percentage of the acid excreted conjugated with glucuronic acid is a necessary consequence of an increased blood level. It should be noted that the term 'blood level' is used in its widest sense, since we are not in a position to decide whether or not the relation between the true blood level and the total amount present in the body is linear. The blood level will depend on the magnitude of the dose of benzoic acid itself or on the dose level and rate of conversion of benzoic acid precursors. It is hoped to investigate the application of these considerations to toluic acid and its precursors.

It has been found that there are considerable differences in the proportions of benzoic acid conjugated with glucuronic acid and glycine by different species. There is also a species difference in the rate of hippuric acid formation (for summary see Williams, 1947). Several suggestions have been put forward to account for the former. Thus Quick (1932) proposed immediate and complete conjugation of glucuronic acid in the dog, followed by partial hydrolysis and glycine conjugation. It can now be seen, however, that the species difference in the glucuronide : hippuric acid ratio can be accounted for solely in terms of the differences in rate of hippuric acid formation. Quick (1931) calculated that the approximate rates of glycine mobilization for man, dog, pig and rabbit were 9·0, 3·5, 15 and 24 mg./kg./hr., respectively. From these values the theoretical percentages of benzoic acid conjugated with glucuronic acid can be calculated, using the expressions (3), (7) and (9) derived in the Appendix, p. 95, taking

Table 2. *Species differences in the degree of conjugation of benzoic acid with glucuronic acid*

Species	Dose level (g./kg.)	Percentage of dose excreted as glucuronide		Rate of glycine mobilization (mg./kg./hr.)
		Calc.	Found	
Dog	0·45	68	66*	3·5*
Man	0·043	9	5†	9·0*
Pig	0·50	41	31‡	15·0*
Rabbit	0·50	24	19§	50·0§

* Quick (1931) † Unpublished results. ‡ Csonka (1924). § Present paper.

g Value for k_g calculated in usual way but only approximate since hippuric acid excretion curve initially linear.
h Experiment concluded before completion of excretion.

the velocity constant for glucuronide formation to be 0·08 as determined in this investigation for the rabbit. The results are given in Table 2. The value for k_h for the rabbit would appear to vary considerably in different individuals. For our present purpose we have used a value of 50 mg. glycine/kg./hr. which we observed for the rabbit (no. 309) used in the actual experiment. It can be seen that there is good agreement between the theoretical and experimentally determined values. It may be noted that some confusion has previously been caused (e.g. Quick, 1932) by the fact that man excretes benzoic acid almost entirely as hippuric acid and the dog mainly as glucuronide, although the rate of mobilization of glycine in both species is low compared with the pig and the rabbit. The difference in dose level accounts for this, since benzoic acid is normally administered to man at a very much lower dose level than to other animals.

Conversion of potential centres

It has been shown that the processes of conversion of benzamide, toluene and benzyl alcohol to benzoic acid follow the kinetics of first-order reactions with velocity constants 0·32, 0·11 and 1·0 respectively. An approximate value for benzaldehyde is 0·33. When the formation of benzoic acid takes place at a rate slower than that at which hippuric acid formation can occur (e.g. Fig. 1 in which the curve showing excretion of hippuric acid (expressed as benzoic acid) with time is exponential) the amount of glucuronide formed is insignificant. Where the rate of benzoic acid production exceeds the maximum rate of its conjugation with glycine (k_h) (i.e. when graph of hippuric acid excretion and time is initially linear) glucuronide formation occurs until the level of benzoic acid falls below that at which maximum glycine conjugation takes place (e.g. Exps. 7, 8, 12, 17 and 18 in Table 1).

The rate of benzoic acid formation is determined by the dose level and the velocity constant of the conversion process. The percentage of the dose excreted as glucuronide depends on the blood level of benzoic acid. Thus in summary it may be stated that the relative amounts of the metabolites of the benzoic acid precursors may be expressed as a function of velocity constants and the dose level at which the precursors are administered (see Appendix and Fig. 6).

Oxidation of benzaldehyde

The velocity constants for this process given in Table 1 are maximum values and approximate. Although the analytical results fitted an exponential curve when the graphical treatment previously described was applied, the deviation was greater than for the other reactions studied. A possible reason for this is the exceptional susceptibility of benzaldehyde to atmospheric oxidation. The substance was distilled *in vacuo* immediately before administration, but an appreciable amount may have been oxidized in the gut before absorption. Since the amount of glucuronide formed was negligible in Exps. 13, 14, 15 and 16, and was taken into account in calculating k_b for Exp. 12 (see Appendix, Fig. 6) it seems likely that the values for k_b are not greatly in error.

Mechanism of toluene oxidation

It is generally assumed that oxidation of the methyl group of toluene takes place as follows:

$$—CH_3 \rightarrow —CH_2OH \rightarrow —CHO \rightarrow —COOH.$$

The results presented above show that the velocity constant for the oxidation of benzyl alcohol is 1·0, while that for benzaldehyde is only 0·33. Thus it is unlikely that both compounds can be intermediate stages in the oxidation of toluene to benzoic acid. Direct evidence has been obtained by Jaffé (1878–9) and Bray, Thorpe & Wood (1949) that benzyl alcohol derivatives are intermediate stages in the oxidation of substituted toluenes, so that it seems reasonable to suggest that benzaldehyde as such is not an intermediate. It has been suggested that hydrate formation, followed by glucuronide conjugation, may precede oxidation or reduction of aldehydes (e.g. Lehmann & Knoefel, 1938; Sammons & Williams, 1946). If hydrate formation occurs with benzaldehyde it is unlikely that conjugation of the hydrate with glucuronic acid takes place, since it is then difficult to explain the observed metabolic results without suggesting a system of reactions for conversion of the conjugated hydrate into hippuric acid, for which there is no experimental evidence.

APPENDIX

When benzoic acid or a precursor is fed, a variable proportion of the dose is excreted as benzoylglucuronide. The amount formed may be expressed as a function of velocity constants and the dose level. In this appendix a general equation is derived. It is assumed that free benzoic acid and a precursor are introduced instantaneously into the body and that the precursor is transformed by a first-order reaction to benzoic acid; benzoic acid is conjugated with glucuronic acid according to a first-order reaction and with glycine at a constant maximum rate k_h. When no free benzoic acid is present and the rate of conversion of the precursor is less than k_h, it is assumed that no glucuronide is formed (see p. 93). A correction for the time during which absorption occurs in actual practice is derived and shown to be relatively small except for very small dose levels.

Symbols. a = amount of precursor in the body at time t; A = amount of precursor in the body at time $t=0$; b = amount of benzoic acid (free and conjugated) in the body at time t; B = amount of benzoic acid (free and conjugated) in the body at time $t=0$; m = amount of conjugated benzoic acid in the body at time t; g = amount of benzoylglucuronide in the body at time t; k_b = first-order reaction velocity constant for

precursor→benzoic acid; k_g = first order reaction velocity constant for benzoic acid→glucuronide; k_h = maximum rate of formation of hippuric acid. Let A precursor and B benzoic acid be introduced into the body instantaneously. Then

$$-da/dt = k_b a \quad \text{and} \quad a = Ae^{-k_b t}, \tag{1}$$

$$b = B + (A - a), \tag{2}$$

and by substitution from equation (1)

$$b = B + A(1 - e^{-k_b t}). \tag{3}$$

When $B > 0$ and/or $-da/dt > k_h$

$$dm/dt = dg/dt + k_h, \tag{4}$$

and

$$dg/dt = k_g(b - m). \tag{5}$$

Substituting from equations (3) and (5) into (4)

$$dm/dt + k_g m = k_g(A + B) - k_g Ae^{-k_b t} + k_h, \tag{6}$$

and on integration

$$m = \left(A + B + \frac{k_h}{k_g}\right)(1 - e^{-k_g t}) + \frac{k_g A}{k_g - k_b}(e^{-k_g t} - e^{-k_b t}). \tag{7}$$

Let the free benzoic acid in the body $(b - m)$ fall to zero at time T, i.e. $(b_T - m_T) = 0$.

Now equations (3) and (7) may be solved for T (by graph). The total amount of glucuronide formed, g_T, may be calculated:

$$g_T = m_T - k_h T = b_T - k_h T. \tag{8}$$

Substituting from equation (3)

$$g_T = A(1 - e^{-k_b T}) + B - k_h T. \tag{9}$$

Correction for time taken for absorption

Let k_{a_1} = rate of absorption of precursor $= A_0/t_a$; k_{a_2} = rate of absorption of benzoic acid $= B_0/t_a$; A_0 = amount of precursor administered; B_0 = amount of benzoic acid administered; t_a = duration of absorption (assumed to be same for A_0 and B_0). During absorption period

$$da/dt = k_{a_1} - k_b a \quad \text{or} \quad a = k_{a_1}/k_b\,(1 - e^{-k_b t}). \tag{10}$$

Let

$$k_{a_1} + k_{a_2} = k_a,$$

then

$$b = k_a t - a. \tag{11}$$

Substituting from (10)

$$b = k_a t - \frac{k_{a_1}}{k_b}(1 - e^{-k_b t}). \tag{12}$$

When

$$db/dt > k_h,$$

then

$$dm/dt = k_g(b - m) + k_h. \tag{13}$$

Assuming $db/dt > k_h$ initially, equation (13), substituting for b from (12), becomes

$$dm/dt + k_g m = k_g\left[k_a t - \frac{k_{a_1}}{k_b}(1 - e^{-k_b t})\right] + k_h. \tag{14}$$

On integration

$$m = \left(\frac{k_h - k_a}{k_g} - \frac{k_{a_1}}{k_b}\right)(1 - e^{-k_g t}) + \frac{k_g k_{a_1}}{k_b(k_g - k_b)}(e^{-k_b t} - e^{-k_g t}) + k_a t. \tag{15}$$

This value for m may be substituted in

$$g = m - k_h t. \tag{16}$$

At time t_a when absorption is just complete, free benzoic acid in the body $= A_0 + B_0 - a - m$, and precursor in the body $= a$. g_T may be derived by application of the same calculation as under conditions of instantaneous absorption (see above). The correction for absorption leads to lower values as shown in Table 3, but the difference between corrected and uncorrected values is only considerable at low dose levels.

Table 3. *Effect (calculated) of absorption period on formation of benzoyl glucuronide*

(Values used for calculation were: $k_g = 0{\cdot}08$ hr.$^{-1}$; $k_h = 140$ mg. hr.$^{-1}$.)

Dose of benzoic acid (g.)	Calculated percentage of glucuronide formed assuming absorption: Instantaneous	Of 2 hr. duration
2·0	33	29
1·0	21	15
0·5	12	7

Fig. 6 shows the relation between dose level and the percentage of benzoic acid conjugated with glucuronic acid after the administration of benzoic acid or a precursor. The values are derived from the expression (9) above, without

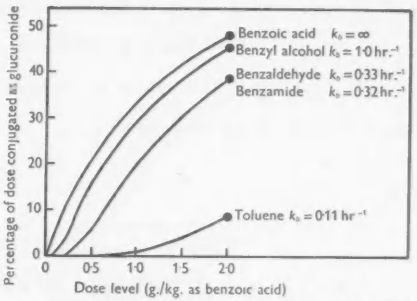

Fig. 6. Variation of glucuronide formation with dose level after administration of benzoic acid and some precursors.

correction for absorption and using the values $k_h = 140$ mg./kg./hr. and $k_g = 0{\cdot}08$ hr.$^{-1}$. In the case of toluene, allowance for excretion via the lungs of part of the dose was made in equations (1) and (2) as follows:

$$(1)\ a = Ae^{-Kt} \quad \text{and} \quad (2)\ b = B + \frac{k_b}{K}(A - a),$$

where K is a first-order reaction velocity constant for the total excretion of toluene by all routes.

SUMMARY

1. The rates of excretion of hippuric acid and benzoylglucuronide by the rabbit after administration of sodium benzoate, benzamide, toluene, benzyl alcohol and benzaldehyde have been studied.

2. The excretion of hippuric acid by rabbits which had received sodium benzoate took place at a constant rate ranging from 115 to 166 mg./hr. Administration of glycine increased this rate.

3. The formation of benzoylglucuronide follows the kinetics of a first-order reaction with velocity constant 0·08 hr.$^{-1}$. The processes of conversion of

benzamide, toluene, benzyl alcohol and benzaldehyde to benzoic acid also follow first-order reaction kinetics with velocity constants 0·32, 0·11, 1·00 and 0·33 hr.$^{-1}$ respectively.

4. Mathematical treatment of the observed kinetics gives expressions from which theoretical values can be derived which are in agreement with experimental observations.

5. The observed kinetics provide an explanation for the different proportions of benzoylglucuronide and hippuric acid excreted after administration of benzoic acid at different dose levels either as sodium benzoate or as precursors.

6. The well-known species differences in the degree of conjugation of benzoic acid with glucuronic acid is also explained.

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REFERENCES

Bray, H. G., Humphris, B. G. & Thorpe, W. V. (1949). *Biochem. J.* **45**, 241.
Bray, H. G., Neale, F. C. & Thorpe, W. V. (1946). *Biochem. J.* **40**, 134.
Bray, H. G., Ryman, B. E. & Thorpe, W. V. (1947). *Biochem. J.* **41**, 212.
Bray, H. G., Ryman, B. E. & Thorpe, W. V. (1948). *Biochem. J.* **43**, 561.
Bray, H. G., Thorpe, W. V. & Wood, P. B. (1949). *Biochem. J.* **45**, 45.
Courtice, F. C. & Gunton, R. W. (1949). *J. Physiol.* **108**, 405.
Csonka, F. A. (1924). *J. biol. Chem.* **60**, 545.
Griffith, W. H. (1938). *J. biol. Chem.* **123**, xlvi.
Griffith, W. H. & Lewis, H. B. (1923). *J. biol. Chem.* **57**, 1.
Jaffé, M. (1878–79). *Hoppe-Seyl. Z.* **2**, 47.
Lehmann, G. & Knoefel, P. K. (1938). *J. Pharmacol.* **63**, 453.
Quick, A. J. (1931). *J. biol. Chem.* **92**, 65.
Quick, A. J. (1932). *J. biol. Chem.* **97**, 403.
Sammons, H. G. & Williams, R. T. (1946). *Biochem. J.* **40**, 223.
Thorpe, W. V. (1950). *Brit. Sci. News*, **3**, 78.
Williams, R. T. (1947). *Detoxication Mechanisms*, p. 95. London: Chapman and Hall.
Wilson, P. W. (1949). *Respiratory Enzymes*, p. 16, ed. by Lardy, H. A. Minneapolis: Burgess Publ. Co.

The Influence of 2:4-Dinitrophenol on the Oxidative Breakdown of Fatty Acids

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Certain substituted phenols have the property of increasing the oxygen uptake of tissue slices. Dodds & Greville (1933) and Elliott & Baker (1935) found that 4:6-dinitro-*o*-cresol in 10^{-5}M concentration accelerated the respiration of slices of several mammalian organs *in vitro*, but other workers found that similar compounds did not have this influence on tissue extracts (for references see Peiss & Field, 1948). Pickett & Clifton (1941) and Clifton (1946) indicated that 2:4-dinitrophenol (DNP) in suitable concentrations inhibited synthetic processes without influencing oxidation, and Hotchkiss (1944) reported that DNP prevented the uptake of inorganic phosphate by respiring yeast. Experiments carried out by Loomis & Lipmann (1948) led them to the conclusion that DNP uncoupled phosphorylation from oxidation in rabbit kidney homogenates. An increased breakdown of glycogen in muscle under the stimulus of DNP has been observed by several workers (for references see Pierce & Field, 1949). Since it is undecided whether phosphorylated compounds take part in the oxidative breakdown of fatty acids, the effect of DNP on this process has been investigated.

EXPERIMENTAL AND RESULTS

All experiments were carried out with liver slices taken from rats maintained on an adequate diet until required. The slices were incubated at 37° in Krebs-Ringer phosphate solution of pH 7·4 for 60 min., unless otherwise stated. The conventional Warburg technique was followed. In all experiments the gas phase was O_2.

Table 1 gives the average results of four experiments in which sodium octanoate was used as substrate. It will be observed that DNP increased the oxygen uptake in the absence and presence of octanoate. In order to establish whether the DNP

concentration used was sufficient to inhibit oxidative phosphorylation it was necessary to test simultaneously a system requiring phosphorylation. A suitable reaction is the conversion of fructose to glucose (Cori & Shine, 1936; Goda, 1937), and Table 2 gives results of experiments on this point. Fructose was estimated by the Seliwanoff reaction as modified by Roe (1934).

Table 1. *Influence of DNP on octanoate oxidation by rat liver slices*

(Krebs-Ringer phosphate, pH 7·4; total vol. 3·4 ml.; time 60 min. Q_{O_2} measured at 37°; based on dry wt. of tissue, which was 14·5–25·7 mg. $Q_{Acac.}$ refers to CO_2 liberated from acetoacetic acid by the aniline citrate technique of Edson, 1935.)

Sodium octanoate	Without DNP		$4·5 \times 10^{-5}$ M-DNP	
	Q_{O_2}	$Q_{Acac.}$	Q_{O_2}	$Q_{Acac.}$
0·01 M	6·5	0·96	9·4	1·37
0	4·4	0·24	5·4	0·36
Differences	2·1	0·72	4·0	1·01

Table 2. *Comparison between fructose and octanoate metabolism of rat liver slices under the influence of DNP*

(Conditions similar to those given in Table 1. Dry weight of slices 20–37 mg.; fructose, 0·011 M; sodium octanoate, 0·01 M.)

Substrate	Without DNP			$4·5 \times 10^{-5}$ M-DNP		
	Q_{O_2}	$Q_{Acac.}$	Fructose after incubation (mg./100 ml.)	Q_{O_2}	$Q_{Acac.}$	Fructose after incubation (mg./100 ml.)
Fructose	8·4	—	106	10·3	—	178
None	6·9	—	—	10·0	—	—
Sodium octanoate	9·6	2·4	—	14·8	4·0	—
None	6·9	0·2	—	10·0	0·6	—

Table 3. *Effect of DNP on the production of β-hydroxybutyrate and acetoacetate from 0·01 M-octanoate*

(Rat liver slices, 1·9 g., were incubated in 30 ml. of Krebs-Ringer phosphate, pH 7·4, in 250 ml. Erlenmeyer flasks. Gas phase O_2. Temp. 37°. Time 90 min.)

$4·5 \times 10^{-5}$ M-DNP	β-Hydroxybutyric acid (mg.)	Acetoacetic acid (mg.)
Present	0·51	3·96
Absent	2·22	1·83

The results show that the liver slices utilized 45% of the fructose in the absence of DNP whilst less than 10% disappeared in its presence. In contrast to this inhibition DNP enhanced the metabolism of octanoate with regard to both oxygen uptake and acetoacetate formation. After incubation with octanoate the reaction products were examined by the steam-distillation technique of Leloir & Munoz (1939). It was found that the amount of substrate disappearing was 25% in the absence of DNP and 27% in its presence. The almost identical utilization of octanoate under either condition suggests that the augmented respiration was connected with some intermediate breakdown product.

It is known from the work of Jowett & Quastel (1935) and Edson (1935) that β-hydroxybutyrate and acetoacetate can be interconverted by slices of mammalian liver. The effect of DNP on the formation of these products was investigated. The estimations were carried out according to the technique of Edson (1935). From the figures given in Table 3 it is quite apparent that DNP altered the ratio between β-hydroxybutyrate and acetoacetate to yield a preponderance of the latter. Similar results were obtained from experiments in which synthetic DL-β-hydroxybutyrate and acetoacetate were incubated with rat liver slices (Table 4). The results show that whilst the enzymic dehydrogenation of β-hydroxybutyrate was enhanced by DNP the reduction of acetoacetate was inhibited.

Table 4. *Effect of DNP on the ratio of DL-β-hydroxybutyrate to acetoacetate*

(Conditions similar to those given in Table 1. Each result is the mean of four experiments. Δ indicates that the results have been corrected for contro lvalues.)

$4·5 \times 10^{-5}$ M-DNP	$\Delta Q_{Acac.}$	
	0·01 M-Sodium DL-β-hydroxybutyrate	0·01 M-Sodium acetoacetate
Present	1·75	−0·24
Absent	1·0	−1·35

Table 5. *Effect of DNP on β-keto-acid production from pyruvate by rat liver slices*

(Sodium pyruvate, 0·002 M. Conditions similar to those given in Table 1. The figures for β-keto-acid were calculated from estimations by the aniline citrate method.)

$4·5 \times 10^{-5}$ M-DNP	Pyruvic acid disappearance (mg./100 ml.)	Pyruvic acid accounted for as β-keto-acid (mg./100 ml.)	Recovery (%)
Present	11·0	12·5	114
Absent	10·5	6·2	59
Present	12·0	13·6	114
Absent	12·0	4·4	37

Since pyruvic acid leads to the same end products as fatty acids when offered to isolated liver (Embden & Oppenheimer, 1913) it was included in this study. Pyruvate determinations were carried out by the method of Bueding, Stein & Wortis (1941). From the amounts of β-keto-acid formed (Table 5) it would appear that, whilst complete conversion of pyruvate into acetoacetate occurred in the presence of DNP, part of the keto-acid was reduced to β-hydroxybutyrate in its absence.

In large-scale experiments rat liver slices were incubated with sodium pyruvate, and ketone bodies were estimated by the gravimetric technique described by Peters & Van Slyke (1932). It was ascertained in control experiments that the final pyruvate concentration was sufficiently low not to interfere with the estimation of ketone bodies. It was also shown that DNP had no influence on the estimation of β-hydroxybutyrate. The results of two experiments are recorded (Table 6).

Table 6. *Effect of DNP on the production of β-hydroxybutyrate and acetoacetate from pyruvate*

(Rat liver slices, 2·5 g., were incubated in 150 ml. Erlenmeyer flasks containing 52 ml. of 0·004 M-sodium pyruvate in Krebs-Ringer phosphate, pH 7·4. Gas phase O_2. Temp. 37°. Time 60 min. Ketone bodies estimated gravimetrically.)

$4·5 \times 10^{-5}$ M-DNP	Pyruvic acid disappearance (mg.)	β-Hydroxybutyric acid formation (mg.)	Acetoacetic acid formation (mg.)	Pyruvic acid accounted for as ketone bodies (mg.)	(%)
Present	11·25	1·1	4·5	9·7	87
Absent	11·25	2·9	2·8	9·83	87
Present	6·7	0·6	3·0	6·15	92
Absent	6·7	1·38	2·26	6·24	93

The results given in Tables 3 and 6 establish that DNP increased the ratio of keto-acid to hydroxy-acid.

DISCUSSION

Confirmation of the hypothesis that phosphorylation participates in the intracellular breakdown of fatty acids has been attempted by several workers. No phosphorylated intermediate product has been isolated. Lehninger & Kennedy (1948) and Cross, Taggart, Covo & Green (1949) observed that fatty acid metabolism proceeds only with the simultaneous oxidation of some member of the citric acid cycle, and this may involve phosphorylation. DNP was found to block oxidation of acetate, butyrate or β-keto-octanoate in the cyclophorase system. On the other hand, Clifton (1946) has shown that acetate or butyrate is oxidized by *Pseudomonas calco-acetica* in the presence of DNP at a physiological pH. Stoppani (1949) found an inhibition of acetate oxidation by yeast in the presence of DNP at the unphysiological pH of 4·5–4·8. The observation in the present work that octanoate and pyruvate oxidation by rat liver slices is not impaired by DNP at a pH near the physiological range, and at a concentration which inhibits fructose metabolism, speaks against a process of oxidative phosphorylation in the fatty acid breakdown. The differing results obtained by Cross *et al.* (1949) can perhaps be explained by the fact that, according to Potter (1946), the system required for octanoate oxidation prepared from rodent livers is very unstable, and addition of DNP often reduces the oxygen uptake of organ homogenates.

In contrast to the inhibition of oxidative phosphorylation processes by DNP sufficient experimental evidence is available to show that reactions requiring anaerobic phosphorylation are not affected by DNP, e.g. the glycogen breakdown in muscle occurs at an accelerated rate in the presence of DNP. Therefore no conclusion can be drawn regarding anaerobic phosphorylations during the chain of reactions linked with the breakdown of fatty acids.

SUMMARY

1. Dinitrophenol (DNP) in a concentration of $4·5 \times 10^{-5}$ M and at pH 7·4 enhanced the respiration of rat-liver slices metabolizing octanoate and pyruvate. The end products in both reactions contained a much greater proportion of acetoacetate than was found in control experiments.

2. Under similar experimental conditions DNP inhibited oxidative phosphorylation of fructose, as indicated by its effect on the aerobic utilization of this sugar.

3. It is suggested that oxidative phosphorylation is not involved in the intracellular breakdown of octanoate or pyruvate.

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REFERENCES

Bueding, E., Stein, M. H. & Wortis, H. (1941). *J. biol. Chem.* **140**, 697.
Clifton, C. E. (1946). *Advanc. Enzymol.* **6**, 269.
Cori, C. F. & Shine, W. M. (1936). *J. biol. Chem.* **114**, xxi.
Cross, R. J., Taggart, J. V., Covo, G. A. & Green, D. E. (1949). *J. biol. Chem.* **177**, 655.
Dodds, E. C. & Greville, G. D. (1933). *Nature, Lond.*, **132**, 966.
Edson, N. L. (1935). *Biochem. J.* **29**, 2082.
Elliott, K. A. C. & Baker, Z. (1935). *Biochem. J.* **29**, 2396.
Embden, G. & Oppenheimer, M. (1913). *Biochem. Z.* **55**, 335.
Goda, T. (1937). *Biochem. Z.* **294**, 259.
Hotchkiss, R. D. (1944). *Advanc. Enzymol.* **4**, 153.
Jowett, M. & Quastel, J. H. (1935). *Biochem. J.* **29**, 2181.
Lehninger, A. L. & Kennedy, E. P. (1948). *J. biol. Chem.* **173**, 753.
Leloir, L. F. & Munoz, J. M. (1939). *Biochem. J.* **33**, 734.
Loomis, W. F. & Lipmann, F. (1948). *J. biol. Chem.* **173**, 807.
Peiss, C. N. & Field, J. (1948). *J. biol. Chem.* **175**, 49.
Peters, J. P. & Van Slyke, D. D. (1932). *Quantitative Clinical Chemistry*, 1st ed., vol. ii (*Methods*), p. 625. Baltimore: Waverly Press.
Pickett, M. J. & Clifton, C. E. (1941). *Proc. Soc. exp. Biol., N.Y.*, **46**, 443.
Pierce, F. T. & Field, J. (1949). *J. biol. Chem.* **180**, 895.
Potter, V. R. (1946). *J. biol. Chem.* **165**, 311.
Roe, J. H. (1934). *J. biol. Chem.* **107**, 15.
Stoppani, A. O. M. (1949). *Nature, Lond.*, **164**, 1096.

A New Volumetric Method for the Determination of Histaminase Activity in Biological Fluids

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Different methods for the estimation of histaminase activity in biological fluids and many different units have been proposed by various workers. The methods described were either chemical (Zeller, 1940; Zeller, Stern & Wenk, 1941; Koloszynski, 1946), manometric (Zeller, Birkhäuser, Mislin & Wenk, 1939; Zeller & Birkhäuser, 1940; Laskowski, 1942; Stephenson, 1943; Swedin, 1943*a*, *b*, 1944) or biological ones (Marcou, Chiriceanu, Cosma, Gingold & Parhon, 1938; Ungar & Parrot, 1939; Albus, 1939; Werle & Effkemann, 1940; Ahlmark, 1944; Anrep, Barsoum & Ibrahim, 1947*a*, *b*; Wicksell, 1949). These methods were applied either to purified histaminase preparations, made from hog kidneys, or to the serum, plasma or placentae of pregnant women. Since the conditions under which these tests were carried out varied widely, the results obtained by various workers can hardly be compared.

In the present investigation a simple volumetric method for the determination of histaminase activity in biological media has been developed and a new histaminase unit proposed. Moreover, an attempt has been made to correlate the results obtained for histaminase activity with chemical, manometric and biological methods by applying these methods simultaneously to a purified preparation of histaminase made from hog kidneys. Finally, the discrepancy in the behaviour of histaminase towards histamine on the one hand and towards cadaverine on the other hand, described and discussed in previous work (Kapeller-Adler, 1944, 1949), was confirmed by means of this new volumetric method. The substance of this paper was communicated at the First International Congress of Biochemistry at Cambridge in August 1949.

METHODS

New volumetric method for the estimation of histaminase in biological media

This method is a modification of Zeller's qualitative indigo test (Zeller *et al.* 1941); it is based on the theory that during the action of histaminase 1 mol. of H_2O_2 is formed for each mol. of histamine oxidized (Kiese, 1940; Zeller, 1938*a*, 1942; Stephenson, 1943; Swedin, 1943*a*, *b*, 1944; Kapeller-Adler, 1949).

A purified preparation of histaminase made from hog kidneys (Kapeller-Adler, 1949) was used. The activity of this preparation was expressed in Laskowski units (L.U.), one unit corresponding to the amount of enzyme which at 37°, pH 7·2, in air, with 1 mg. of histamine dihydrochloride as substrate, utilizes 1 μl. O_2/min. (Laskowski, 1942).

Reagents required: (1) M/15-phosphate buffer (Sörensen) pH 7·2. (2) Histamine solution in M/15-phosphate buffer, containing 10 mg. of histamine dihydrochloride/ml. (3) Indigo disulphonate solution. Indigo Carmine (200 mg.) is dissolved in 300 ml. water and the solution stored in a brown bottle. (4) 0·002 N-$KMnO_4$ solution. (5) $CHCl_3$.

Procedure. To increasing amounts of enzyme solution, placed in Pyrex test tubes (0·1–1 ml. of a solution of the histaminase preparation in phosphate buffer, corresponding to 0·0025–0·025 L.U.), 1 ml. of the indigo solution and 0·1 ml. of the histamine solution are added and the volume made up with phosphate buffer to 4 ml. $CHCl_3$ (1 drop) is added as a

preservative. O_2 is passed through the fluid for 1 min., the test tube is closed with a rubber stopper, the solution well mixed by shaking and incubated for 24 hr. at 37°. A control for each individual test without the addition of histamine is always carried out simultaneously with the test. After 24 hr. the controls and the tests containing an excess of the indigo compound are titrated with 0·002 N-$KMnO_4$ until the end point of the titration is reached, when the blue colour has just disappeared and a pink colour just appears and persists for a few seconds. The amount of $KMnO_4$ utilized in the assay is subtracted from the amount taken up by the control. The difference indicates the amount of H_2O_2 formed by the action of histaminase on histamine and thus the enzyme activity.

Estimation of histaminase activity of serum by the volumetric method

Add to 1 ml. of non-haemolysed serum 2·5 ml. of phosphate buffer pH 7·2, 0·1 ml. of the histamine solution, 0·5 ml. of the indigo solution and 1 drop of $CHCl_3$. Bubble O_2 through for 1 min. and proceed as above. A control serum without the addition of the histamine solution is worked up simultaneously with the test serum. If possible, controls and test sera should be done in triplicate.

Estimation of histaminase activity in serum by a biological test

The biological test of Anrep *et al.* (1947*a*, *b*) has been slightly modified. Place 1 ml. of serum in a small conical flask in an incubator (37°) for 5 min. Add 0·3 ml. of an aqueous solution of histamine acid phosphate containing 3·6 μg. of histamine base, and incubate for 30 min. at 37°. After that time dilute rapidly with 4·7 ml. of Ringer solution and heat the fluid to 80°. After cooling, the solution is assayed on the isolated guinea pig ileum against the control solution.

Control test. To 1 ml. of serum 4·7 ml. of Ringer solution are added and the fluid is heated to 80°. After cooling 0·3 ml. of histamine acid phosphate solution, containing 3·6 μg. of histamine base is added, the mixture incubated for 30 min. at 37° and then assayed on the guinea pig ileum.

Anrep's modified biological test was also used for the estimation of histaminase activity in histaminase preparations. More histamine (6·0 μg. instead of 3·6 μg. of base) was added to enzyme solutions containing more than 0·0125 L.U., and to more active sera.

RESULTS

Simultaneous application of the new volumetric method and Anrep's biological test to histaminase preparations. Fig. 1, *a* and *b*, show that when both tests were simultaneously applied to increasing amounts of a purified histaminase preparation the volume of permanganate taken up and the amount of histamine inactivated was directly proportional to the amount of enzyme present. The volume of permanganate utilized was 6% more than might have been expected theoretically from the oxygen consumption.

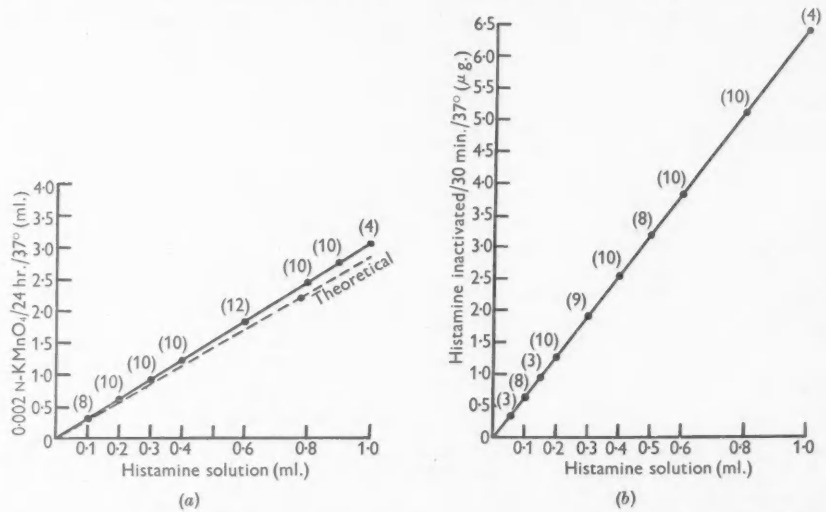

Fig. 1. Effect of increasing amounts of histaminase from hog kidneys on histamine. (*a*) Volumetric test. (*b*) Biological test. The number in brackets is the number of separate estimations on which the average result shown was calculated.

New histaminase unit. The results of the new volumetric test are expressed in terms of a new histaminase unit. This unit, conveniently called a permanganate unit (P.U.), represents the amount of enzyme which, after an incubation of 24 hr. at 37° and pH 7·2, in an atmosphere of oxygen with 1 mg. of histamine hydrochloride as substrate and with an

aqueous solution of indigo disulphonate, takes up 0·1 ml. 0·002N-potassium permanganate.

If this quantitative indigo test is carried out in air only about 35% of the histaminase activity in an atmosphere of oxygen is obtained. Table 1 shows

Table 1. *Activity of one permanganate unit* (P.U.)

	Histamine inactivated (μg./hr.)	O_2 taken up (μl./hr.)
Theoretical	0·463	0·053
Observed	0·417	0·050
% of theory	90·00	94·00

Table 2. *Comparison of permanganate units with units depending on oxygen consumption*

	P.U.
Laskowski's unit	1200
Zeller's unit	224
Stephenson's unit	120
Torantil unit	50

the rate of histamine destruction and the rate of oxygen consumption by one permanganate unit compared with values calculated on the theory that 1 mol. of histamine is equivalent to 1 mol. of hydrogen peroxide and 0·5 mol. of oxygen. 1 P.U. thus corresponds to the destruction of 0·46 μg. of histamine and to an uptake of 0·05 μl. oxygen/hr. The results obtained were 10% less for the histamine inactivation and 6% less for the oxygen uptake that might have been expected theoretically.

In Table 2 a comparison is made between the permanganate unit and units calculated on the basis of oxygen consumption.

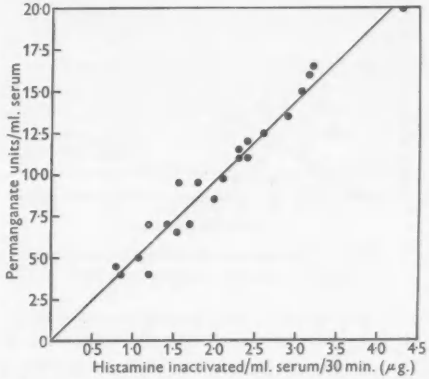

Fig. 2. Relationship between the volumetric test and the biological test when both tests were applied simultaneously to pregnancy sera.

Estimation of histaminase activity in sera. When the new volumetric test and Anrep's biological test were applied to non-pregnancy sera no histaminase activity was detected.

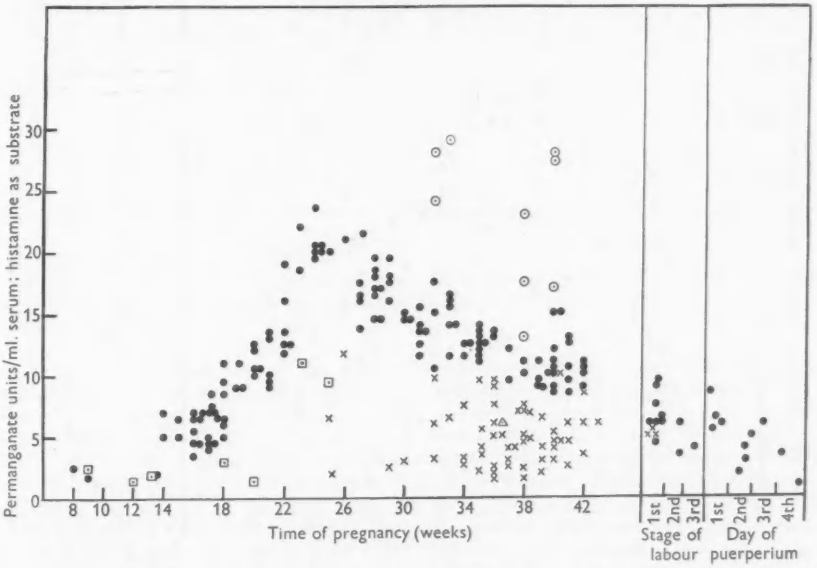

Fig. 3. Histaminase content of 1 ml. of serum of women at various stages of normal pregnancy, ●; twin pregnancy, ⊙; triplet pregnancy, △; toxaemic pregnancy, ×; and threatened abortion, ⊡. Results obtained during labour and in the puerperium are also shown.

The results found when both methods were simultaneously used for the investigation of sera from

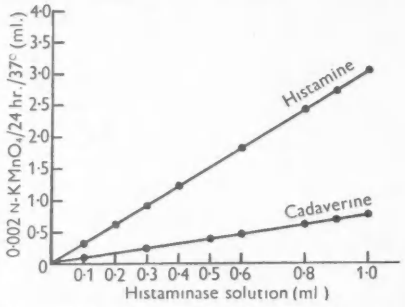

Fig. 4. Effect of histaminase on histamine and cadaverine determined by the volumetric method.

twenty-two women in various stages and conditions of pregnancy are shown in Fig. 2, the values obtained by the biological test being plotted against the values by the volumetric method. This Figure shows that a reasonably good agreement between the two methods was obtained. The central line represents the relationship between the two tests calculated from results obtained when both methods were simultaneously applied to purified enzyme preparations.

In view of the good agreement between the chemical and the biological test it was decided to use only the volumetric method for all subsequent estimations of histaminase activity. In Fig. 3 results are recorded which were obtained when this method was applied to the sera of about 230 pregnant women, women in labour and in the puerperium. The abscissa indicates weeks of pregnancy, stage of labour and days of the puerperium, and the ordinate the P.U./ml. of serum. According to the results obtained histaminase activity becomes apparent in the serum at the end of the 2nd month of pregnancy, reaches its maximum between about the 22nd and 26th week of gestation, and somewhat decreases in the 7th month of pregnancy to remain

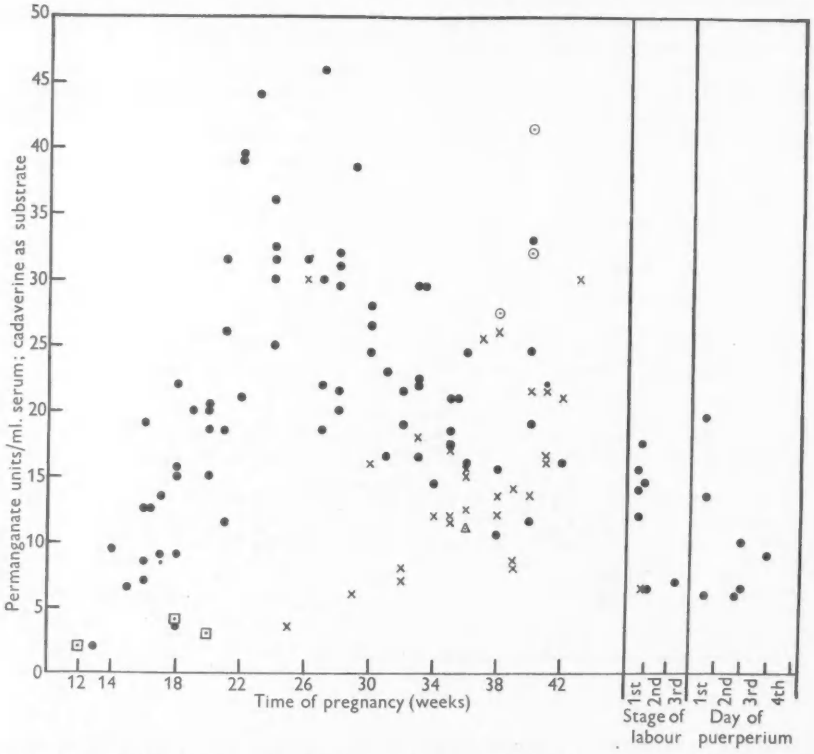

Fig. 5. Effect of serum histaminase on cadaverine in normal pregnancy, ●; twin pregnancy, ⊙; triplet pregnancy, △ toxaemic pregnancy ×; and threatened abortion, ⊡.

more or less stationary until delivery. During labour, and still more rapidly in the puerperium, histaminase activity tends to decrease. Abnormal results were found in three types of cases. Significantly low results were obtained in cases of pre-eclamptic toxaemia, the severe cases showing a very small histaminase activity. Significantly high results were found in most of the cases of twin pregnancy. An unexpected low result was encountered in a case of triplet pregnancy. In several cases of threatened abortion the estimated histaminase activity was exceedingly low.

Figure that histaminase preparations from hog kidneys are four times more active on histamine than on cadaverine.

The results shown in Fig. 5 were obtained when the volumetric test with cadaverine as substrate was applied to some of the sera of pregnant women whose histaminase action on histamine was presented in Fig. 3.

On comparing Figs. 3–5 one finds a discrepancy in the behaviour of serum histaminase and of histaminase preparations from hog kidneys towards cadaverine. Whereas the latter preparations are four

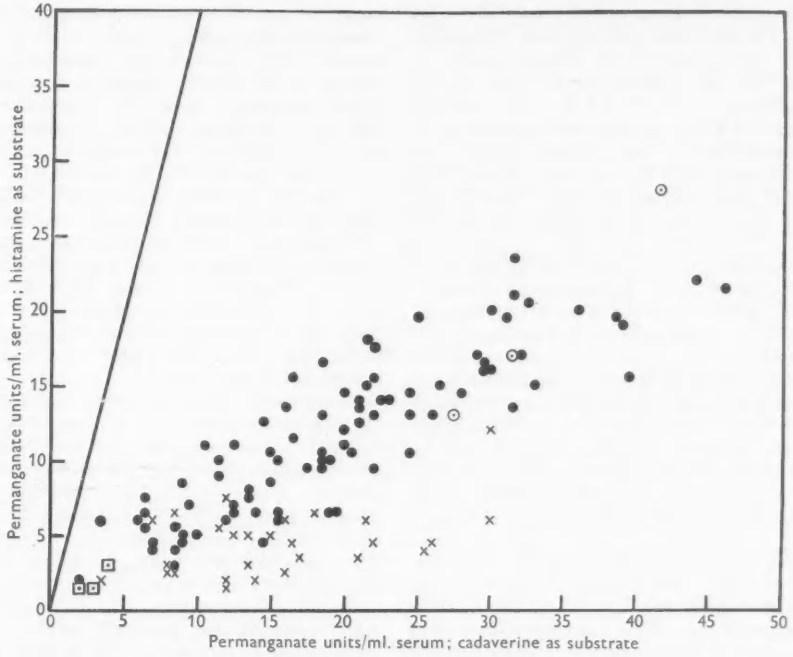

Fig. 6. Relationship between the effect of serum histaminase on histamine and that on cadaverine. Normal pregnancy, ●; twin pregnancy, ⊙; toxaemic pregnancy, ×; and threatened abortion, ⊡. The line indicates the relationship in histaminase preparations from hog kidneys.

Effect of histaminase on cadaverine. In view of contradictory observations concerning the action of histaminase on cadaverine (Zeller, 1938*a*, *b*; Zeller, Birkhäuser, Mislin & Wenk, 1939; Zeller, Schär & Stählin, 1939; Zeller, Stern & Wenk, 1940; Kapeller-Adler, 1944, 1949) the volumetric method was also used to study this reaction. Fig. 4 shows results obtained with various purified preparations of histaminase from hog kidneys with known activity acting on 1 mg. of histamine-dihydrochloride and 1 mg. of cadaverine dihydrochloride in parallel assays. It follows from this

times more active on histamine than on cadaverine (Fig. 4), serum histaminase is more active on cadaverine than it is on histamine. More striking results were obtained in cases of pre-eclamptic toxaemia, confirming previous observations (Kapeller-Adler, 1944). Whereas a significantly decreased enzymic activity was encountered in the sera when histamine was used as substrate, normal results were obtained in most of the toxaemic cases with cadaverine as substrate.

In Fig. 6 the values for P.U./ml. serum with cadaverine as substrate and those with histamine as

substrate are plotted against each other. If the behaviour of histaminase in pregnancy sera towards cadaverine had been identical with that of histaminase preparations from hog kidneys the results would be expected to fall on or near the line drawn on the left side of the diagram.

DISCUSSION

For the quantitative estimation of histaminase activity in biological fluids only biological methods could be successfully used up to now. Chemical methods, mainly published by Zeller (1940) and Zeller and his co-workers (1941), did not prove satisfactory in the investigation of sera. Koloszynski (1946) tried to transform Zeller's qualitative indigo test into a colorimetric one by extracting the excess indigo dye with a mixture of acetone and saturated ammonium sulphate and measuring the dye in a colorimeter. In an attempt to repeat this work in this laboratory it was found, however, that the indigo dye did not readily dissolve in the mixture mentioned above, and very unreliable results were obtained.

The manometric method for the estimation of histaminase activity in pregnancy serum (Zeller & Birkhäuser, 1940) was not any more satisfactory. The oxygen uptake in the controls without the substrate was always very high, sometimes even higher than in the tests containing histamine as substrate. The results shown in Table 1 of the present paper furnish the explanation for the shortcomings of Zeller's manometric method. The amounts of histaminase present in pregnancy sera are far too small to show an oxygen consumption which could be accurately measured in Warburg manometers (0·25–1·25 μl. oxygen/hr.).

The best known biological method for the determination of histaminase activity in biological media is that published by Ahlmark (1944). This method, being very sensitive, allows the estimation of very small amounts of histaminase in plasma. It has, however, the disadvantage of being very tedious and requiring large amounts of material (13–17 ml. of plasma from each patient). Besides, one serious criticism has to be made when this method is used for the determination of histaminase activity in not very active plasmas. Ahlmark incubates these plasmas with histamine at 37° for up to 22 hr. without a preservative. Under these circumstances the possibility cannot be excluded that the loss of histamine might be due not to the activity of histaminase but to bacterial influence. Moreover, Anrep *et al.* (1947*a*) point out that the histaminolytic effect of non-pregnancy plasma, observed by Ahlmark, is not abolished by raising the temperature to 80° and cannot, therefore, be attributed to histaminase activity. The biological method of Anrep and his co-workers (1947*a*, *b*) in the modification mentioned above is simple and gives satisfactory results. Its main disadvantage is that it depends on animal material. Wicksell (1949) tried to simplify Ahlmark's method, but his method still remains rather complicated and does not offer any advantages over the modified Anrep method.

As to the results obtained in the present work it may be said that in normal pregnancy cases they agree quantitatively with those obtained by Ahlmark (1944). Whereas in cases of severe pre-eclamptic toxaemia a significant decrease of serum histaminase was shown with the new quantitative indigo method, Ahlmark found in cases of pregnancy toxaemia, independent of the severity of cases, normal, diminished and increased histaminase activity in the plasma. Anrep investigated twelve cases of severe pre-eclamptic toxaemia in the 35th to 41st week of gestation. Of these cases eight showed a normal histaminase activity and four a reduced one. He did not, however, find any increased histaminase activity. In cases of threatened abortion all methods indicate a significant decrease in histaminase activity. No extensive data are available in the literature referring to the increased histaminase activity in the serum of women with twin pregnancy, observed in this work. Swanberg (1948) mentions one case of a mother of twins in labour whose blood histaminase was a little higher than that of other women in labour.

In agreement with Anrep no other condition has so far been encountered in which histaminase could be detected in human blood. Nor has histaminolysis been found to occur in the blood of pregnant or non-pregnant animals. These findings emphasize the importance of the presence of histaminase in the blood during human pregnancy. It may perhaps be a prophylactic measure against a potential histamine intoxication. More work will have to be done on cases with pre-eclamptic toxaemia to find out the significance of the decreased values for serum histaminase in women with severe pre-eclamptic toxaemia.

Whereas Best, who discovered histaminase in 1929, considered this enzyme to be specific for histamine, Zeller, 1938*a*, *b* and Zeller and his co-workers in a number of papers (1939, 1940) advanced evidence that histaminase also acts on diamines such as cadaverine, putrescine and agmatine. They therefore replaced the term of histaminase by that of diamine oxidase. Though most of the subsequent workers accepted this suggestion of Zeller's school, some doubts were cast on the identity of histaminase and diamine oxidase when Zeller's qualitative indigo test was applied to the investigation of sera from women with pre-eclamptic toxaemia, and of placental extracts (Kapeller-Adler, 1944). The question was, therefore, reinvestigated (Kapeller-Adler, 1949).

From the results obtained it appeared probable that histaminase is an enzyme with various modes of behaviour towards histamine, for which it shows the greatest affinity, and towards diamines such as cadaverine, putrescine and agmatine. In the present work it has been demonstrated that histaminase preparations made from hog kidneys and serum histaminase of pregnant women differed in the relation between their actions on histamine and cadaverine. This discrepancy in the behaviour of histaminase, depending on the origin of the enzyme, suggests that the effect of histaminase on cadaverine, unlike that on histamine, is a non-specific one. In this connexion it should be mentioned that Blaschko (1949), in a recent communication, has intimated that diamine oxidase was active only on diamines up to a chain length of eight carbon atoms. Diamines with seven or more carbon atoms were, however, oxidized by amine oxidase. This enzyme did not act on diamines with six or less carbon atoms.

On account of all these observations it appears to be reasonable to drop the name of diamine oxidase and to re-establish that of histaminase for the enzyme which specifically acts on histamine.

SUMMARY

1. A new simple volumetric method for the estimation of histaminase activity in biological media is described and a new histaminase unit, called permanganate unit (P.U.), is proposed. The effect of this new unit is compared with that of other known histaminase units. 1 P.U. inactivates 0·46 μg. of histamine/hr. with the uptake of 0·05 μl. oxygen.

2. This new method was applied to the investigation of histaminase activity in non-pregnancy sera and in sera during normal and abnormal pregnancy. The results obtained are discussed.

3. The discrepancy in the effects of histaminase on histamine and cadaverine is well shown when the new volumetric method is applied to the investigation of histaminase preparations from hog kidneys and serum histaminase.

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REFERENCES

Ahlmark, A. (1944). *Acta physiol. scand.* **9**, 1.
Albus, G. (1939). *Klin. Wschr.* **18**, 858.
Anrep, G. V., Barsoum, G. S. & Ibrahim, A. (1947*a*). *J. Physiol.* **106**, 379.
Anrep, G. V., Barsoum, G. S. & Ibrahim, A. (1947*b*). *J. Obstet. Gynaec.* **54**, 619.
Best, C. H. (1929). *J. Physiol.* **67**, 256.
Blaschko, H. (1949). *1st Int. Congr. Biochem. Abstr.* p. 565.
Kapeller-Adler, R. (1944). *Biochem. J.* **38**, 270.
Kapeller-Adler, R. (1949). *Biochem. J.* **44**, 70.
Kiese, M. (1940). *Biochem. Z.* **305**, 22.
Koloszynski, E. W. (1946). *J. Obstet. Gynaec.* **52**, 486.
Laskowski, M. (1942). *J. biol. Chem.* **145**, 457.
Marcou, J., Chiriceanu, D., Cosma, G., Gingold, N. & Parhon, C. C. (1938). *Pr. méd.* **46**, 371.
Stephenson, N. R. (1943). *J. biol. Chem.* **149**, 169.
Swanberg, H. (1948). *Acta physiol. scand.* **16**, 83.
Swedin, B. (1943*a*). *Nord. Med.* **17**, 513.
Swedin, B. (1943*b*). *Acta med. scand.* **114**, 210.
Swedin, B. (1944). *Ark. Kemi Min. Geol.* **17** A, 27.
Ungar, G. & Parrot, Y. L. (1939). *C.R. Acad. Sci., Paris*, **208**, 598.
Werle, E. & Effkemann, G. (1940). *Arch. Gynaek.* **170**, 82.
Wicksell, F. (1949). *Acta physiol. scand.* **17**, 359.
Zeller, E. A. (1938*a*). *Helv. chim. Acta*, **21**, 880.
Zeller, E. A. (1938*b*). *Helv. chim. Acta*, **21**, 1645.
Zeller, E. A. (1940). *Helv. chim. Acta*, **23**, 1509.
Zeller, E. A. (1942). *Advanc. Enzymol.* **2**, 93.
Zeller, E. A. & Birkhäuser, H. (1940). *Schweiz. med. Wschr.* **70**, 975.
Zeller, E. A., Birkhäuser, H., Mislin, H. & Wenk, M. (1939). *Helv. chim. Acta*, **22**, 1381.
Zeller, E. A., Schär, B. & Stähli n, S. (1939). *Helv. chim. Acta*, **22**, 837.
Zeller, E. A., Stern, R. & Wenk, M. (1940). *Helv. chim. Acta*, **23**, 3.
Zeller, E. A., Stern, R. & Wenk, M. (1941). *Schweiz. med. Wschr.* **71**, 1349.

The Isolation of a Toxic Substance from Agenized Wheat Flour

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In 1937, Melnick & Cowgill reported the occurrence of 'running fits' in dogs when gliadin was the sole source of protein in the diet. Similar symptoms were not produced when gliadin was replaced by other proteins. The authors concluded that a toxic substance accumulated in the body when gliadin was fed. Wagner & Elvehjem (1944) examined the effect of adding various supplements to a gluten-rich diet and also concluded that 'running fits' were caused by a toxic substance in wheat products rather than by any nutritional deficiency.

In 1946, Mellanby showed that wheat flour which had been 'improved' by treatment with nitrogen trichloride (agenized flour) produced symptoms in dogs similar to running fits; a flour which had not been agenized was not toxic.

Much wheat is grown to-day which, if freshly milled, produces flour which is unsuitable for use in baking. If, however, such wheat is allowed to become aged or 'oxidized' by means of long periods of storage and is then milled, the resulting flour has greatly improved baking properties. This 'oxidizing' process can be more quickly brought about by the use of various oxidizing agents or 'improvers' on the flour. Nitrogen trichloride ('agene') was introduced as an artificial ageing agent by Baker (1921), hence the term agenized flour. The use of nitrogen trichloride has gradually spread, both in this country and in the United States, until in 1946 it was estimated that over 90% of the flour milled in England was treated with nitrogen trichloride. A review, in which the use of oxidizing agents in the treatment of flour is discussed, has been published by Blish (1945).

When agenized flour forms a substantial part of the diet of dogs, the animals become progressively more restless over a period of days and eventually develop epileptiform fits. If the diet is replaced at this stage by an exactly similar diet containing untreated flour the animals recover, but if the diet of agenized flour is continued the animals become progressively worse and die. Similar fits cannot be produced by unagenized flour (Mellanby, 1946) so that it must be assumed that the gliadin used by Melnick & Cowgill (1937) had been treated with agene.

There are striking differences in the susceptibility of different species to the toxic action of agenized flour. Mellanby (1947) showed that the ferret was nearly as sensitive as the dog, but that rats and mice did not develop fits. Newell, Erickson, Gilson, Gershoff & Elvehjem (1947) showed that guinea pigs and chicks were not susceptible, and Radomski, Woodard & Lehman (1948) showed that rabbits developed typical fits.

When the present investigation was begun it was already known (Mellanby, 1947) that the toxicity of agenized flour was associated with the gluten fraction, and that other proteins could also be rendered toxic by treatment with nitrogen trichloride (Moran, 1947). In view of the widespread use of agenized flour it became important to isolate and determine the nature of the toxic substance present in wheat flour. The present communication is concerned with the method used in the isolation of the substance and with its properties. A preliminary account of this work has already been published (Campbell, Work & Mellanby, 1950). The ferret has been used as the test animal and the toxic dose has been regarded as that quantity of material which would produce a typical epileptiform fit.

While the present work was in progress Bentley, McDermott, Pace, Whitehead & Moran (1949*b*) isolated from zein treated with nitrogen trichloride a crystalline substance which was toxic to rabbits. As indicated in the appropriate section this material appears to be identical with the material isolated by us from wheat flour.

MATERIALS AND METHODS

As an example of the methods used in the isolation of the toxic substance from agenized wheat flour, the treatment of a typical batch by the method finally adopted is described. Details showing the course of the isolation, the losses involved and the toxic dose at each stage are to be seen in Table 1. It should be emphasized that the figures quoted in this table are in some cases only an approximation; for although the losses in toxicity and the variation in the toxic dose which resulted from each step were determined, it was not possible to carry out each determination during the course of the fractionation of the same sample of wheat flour owing to the large quantities of material required for each

biological test and the lack of a micro-test. The figures in the table, therefore, represent a composite picture built up from results obtained during the course of several fractionations, but they do represent the course of the fractionation in a qualitative manner. It should be further emphasized that while we have every reason for confidence in the biological test employed, i.e. the production of an epileptiform fit in a ferret, no extensive experiments have been carried out to determine the mean toxic dose of any one preparation over a series of animals, so that the figures for the toxic dose quoted are again only an approximation. The fact that it has been necessary to carry out the biological test by the addition of the test substance to the normal food of the animal rather than by injection also contributed to the difficulties.

Separation of gluten from flour and treatment with pepsin and trypsin

Flour (17·7 kg.) which had been treated with 11 g. NCl_3 by Wallace and Tiernan Co. Ltd., Chiswick, London, was placed in a muslin bag in small batches and kneaded with running water until all the water-soluble material had been removed. The insoluble gluten was then mixed with 13 l. of distilled water containing 30 ml. of conc. H_2SO_4. Pepsin (40 g.) (pepsin powder, B.P., supplied by Chas. Zimmerman and Co. Ltd.) was added and the whole incubated at 37–38° with constant stirring for 24 hr. The pH of the suspension was then brought to 8·1–8·4 by the addition of 5 N-NaOH and trypsin (40 g., supplied by British Drug Houses Ltd.) was added. A little toluene was added to the suspension which was incubated as before for 48 hr. The pH of the suspension, which tended to fall during the early part of the digestion, was checked every few hours and adjusted to 8·1–8·4 by the addition of 2 N-NaOH. If the pH at any time fell below 7·0 it was assumed that the trypsin had been destroyed and a further 40 g. of trypsin were added. The suspension was then filtered and the residue washed with distilled water. The total volume of the digest and washings was about 14 l. which contained approximately 150 g. N and was equivalent to 101 toxic doses (fraction *A*, Table 1).

Preliminary experiments on enzymic digest

Before attempting to develop a method of fractionation, some general knowledge of the properties of the toxic substance had to be obtained.

Heat stability. A sample of digest was boiled for 1 hr. at pH 5·0 and the precipitated protein was removed; activity was not decreased.

Acid stability. Samples of digest were boiled with 6 N-H_2SO_4 for 1, 4 and 18 hr. The SO_4^{--} was removed as $BaSO_4$ and each sample was biologically tested. Up to 4 hr. there was less than 20% loss in activity (a 20% loss was considered to be the minimum detectable without recourse to treatment of a group of animals and statistical analysis of the result). The sample heated for 18 hr. retained less than half of the activity of the digest.

Alkali stability. A sample of digest was heated for 1 hr. with excess 2 N-$Ba(OH)_2$ and Ba^{++} removed as $BaSO_4$. No loss in activity could be detected.

Dialysis. A sample of digest was dialysed against successive portions of distilled water until no more material dialysed. The dialysate and residue possessed the same toxicity relative to their N content, but only 25% of the activity was retained in the dialysis sac.

Liberation of NH_2 groups by acid. The Van Slyke HNO_2 method was used to estimate the apparent NH_2-N:total N ratio. When the digest was boiled with 6 N-H_2SO_4 for 4·5 hr. the proportion was increased from 27 to 54%, while boiling with conc. HCl for 3·5 hr. increased the ratio to 61%. Thus a greater degree of hydrolysis in a shorter time was effected with HCl than with H_2SO_4. The loss in activity after boiling for 3·5 hr. with conc. HCl was slightly greater than after boiling for 4·5 hr. with 6 N-H_2SO_4, but the average molecular weight as indicated by the ratio NH_2:total N was smaller after HCl hydrolysis and this was accordingly adopted as a standard hydrolytic procedure.

Dialysis of enzymic digest

The enzymic digest (*A*, 14 l., 150 g. N) was concentrated under reduced pressure on a water bath (40–60°) to approximately 6 l. and was dialysed in cellophan sacs against 15 l. of distilled water at approximately 5°. Half the dialysate was removed after the first 2 days and replaced with an equal volume of distilled water. This process was repeated three times, after which the whole of the dialysate was replaced by distilled water three times. At the end of this time the amount of dialysable material which could be collected in 2 days did not represent more than 1% of the total N originally contained in the sacs. The combined dialysates were concentrated to 5·5 l. under reduced pressure at 40–60°. The total N in the combined concentrated dialysates was 125 g. (fraction *B*).

Acid hydrolysis of the dialysate

The dialysate (*B*, Table 1) was hydrolysed in four batches with conc. HCl by boiling 1·5 l. with 6 l. of 10 N-HCl for 3·5 hr. under a reflux condenser. Most of the acid was subsequently removed by evaporation of the hydrolysate under reduced pressure on a water bath at 40°. The residual gum was diluted with a little water and reconcentrated to remove a further quantity of HCl. The combined filtered hydrolysates were diluted to 27 l. with distilled water and treated with the anion-exchange resin 'Deacidite E' (obtained from The Permutit Co.). The resin (approx. 2 kg.), after being well washed with distilled water, was activated by allowing it to stand overnight in 5 l. of 2 N-NaOH, washed with 10 l. of distilled water by decantation, loaded into a porcelain pipe (11·5 × 75 cm.), and washed with distilled water until 100 ml. of effluent required less than 3 ml. of 0·02 N-HNO_3 to neutralize it to methyl orange. The acid hydrolysate solution was added to the column and the effluent collected. When the pH of the effluent fell below 5·5 the column was washed clear of hydrolysate with distilled water and was regenerated with 0·5 N-NaOH (30 l.) and washed as before with distilled water. The remaining acid hydrolysate was then added together with the acid effluent obtained from the first column. The combined effluents were concentrated under reduced pressure on a water bath to approx. 12 l. so that the concentration of N was about 10 mg. N/ml. (total N = 118 g., fraction *C*, Table 1).

Electrodialysis of acid hydrolysate

After decolorizing the neutral acid hydrolysate (*C*) by boiling with a little activated charcoal, the hydrolysates were subjected to electrodialysis after the method of Cox, King & Berg (1929). The apparatus consisted of a Perspex box divided into three sections each of which had a total

Table 1. *Stages in the isolation of toxic substance from wheat flour*

Process	Toxic dose	Total N available	No. of doses
Gluten	—	220 g.	—
Treated with 11 g. NCl_3	1·6 g. N	220 g.	140
After enzymic digestion (*A*)	1·5 g. N	Approx. 150 g.	101
After dialysis against distilled water (*B*)	1·5 g. N	125 g.	83
After hydrolysis with conc. HCl (*C*)	2·3 g. N	118 g.	50*
After electrodialysis	700 mg. N	35 g.	—
After removal of crystalline material (*D*)	500 mg. N	25 g.	50
After removal of aromatic amino-acids on charcoal (*E*)	460 mg. N	23 g.	—
After fractionation on Zeo-Karb†	—	—	—
Total of all active fractions	170 mg. N	5·5 g.	32
After removal of crystalline material	130 mg. N	4 g.	32
Most active fraction from column	96 mg. N	2·2 g.	23
After removal of crystalline material (*F*)	56 mg. N	1·3 g.	23
After fractionation on paper column‡ (*G*)	4 mg. N	64 mg.	16
Crystallization from aqueous ethanol	3 mg. solid	—	12

* Acid hydrolysis + electrodialysis caused a loss of one-third of the toxic substance. This is assumed to occur during acid hydrolysis during which stage there is little loss of N, thus the toxic dose goes up.

† For fractionation from Zeo-Karb column, see Table 3.

‡ For fractionation from paper column, see Table 4.

volume of 4 l. The most satisfactory membranes were prepared by treatment of animal parchment (plain skins from Witherby and Co., 326 High Holborn, London, W.C. 1) with 10% (w/v) formaldehyde for 5 hr. at 20°. These membranes had great mechanical strength and electro-endosmosis was minimal. The centre compartment of the box was fitted with a mechanical stirrer and the outer walls of the electrode compartments (platinum electrodes) were cooled (for a review of electrodialysis methods cf. Svensson, 1948). The hydrolysate (3–4 l.; 30–40 g. N) was poured into the centre compartment of the box and the end compartments were filled with distilled water. The electrodes were then connected to a 230 V., d.c. supply main with a 100Ω variable resistance in the circuit. The current rose rapidly to 2·5–2·8 amp., the pH of the anode compartment fell and that of the cathode compartment rose. The duration of the dialysis depended on the amount of N initially added to the centre compartment and on the membranes, but for 40 g. N there was usually a fall in current to about 1·8 amp. in 24 hr. At the end of this time the contents of the end compartments were withdrawn (all but about 200 ml.), and were replaced with distilled water. Within the next 4–8 hr. the current fell to 0·4 amp. and then remained steady. At this point electrodialysis was considered to be complete.

Table 2. *Distribution of nitrogen during electrodialysis of acid hydrolysate*

(Results expressed as g. N.)

	Compartment		
	Cathode	Centre	Anode
Mean values for four runs	9·6	5·0	14·2
Percentage of total N	33	17	50

While the major part of the toxic substance remained in the centre compartment during dialysis a small amount travelled to the cathode compartment so that the contents of the latter were collected, concentrated under reduced pressure and re-electrodialysed together with sufficient of the anode solution to bring the pH to 5·5. The anode solution was not toxic. The total amount of N in the combined neutral fractions from the electrodialysis was 35 g., the toxic dose containing 700 mg. N. The combined neutral fractions were concentrated under reduced pressure on a water bath until crystallization took place. The crystalline material was removed by filtration and the concentration repeated until no further crystallization took place. By this means the total N in the solution was reduced to 25 g. and the toxic dose to the equivalent of 500 mg. N (fraction *D*, Table 1). The distribution of N during four electrodialyses is shown in Table 2.

Removal of aromatic amino-acids with charcoal

The aromatic amino-acids were removed by adsorption on activated charcoal. The charcoal was first treated with acetic acid according to the method of Tiselius, Drake & Hagdahl (1947) and then used as described by Schramm & Primosigh (1943). The filtrate (*D*) was treated in batches such that each batch contained approximately 4 g. N. Each batch was first diluted with 5% (v/v) acetic acid until the concentration of N was 1 mg. N/3 ml. Preliminary experiments on a pilot scale had shown that less than 20% of the N was in the form of aromatic amino-acids and this figure was used as a basis for the calculation of the amount of charcoal required; 1 g. of charcoal being required to adsorb 1 mg. of aromatic amino-acid N. The required amount of charcoal in a suspension in 5% acetic acid was poured into a Büchner funnel and washed with further quantities of 5% acetic acid. When the charcoal was nearly dry the solution of amino-acids was added and the effluent collected at a rate of approximately 5 l./hr. The charcoal was then washed with a volume of 5% acetic acid equivalent to 25 ml./g. of charcoal present, at a rate of approximately 10 l./hr. The effluent and washings were combined. The total amount of charcoal required to treat the 25 g. of N was 5 kg. and the total effluent amounted to 200 l. (fraction *E*).

Fractionation on Zeo-Karb 215 column

The effluent (*E*) from the charcoal was divided into three parts (approx. 10 g. N each) for fractionation (Partridge, 1949) on a cation-exchange column of Zeo-Karb 215 (obtain-

able from The Permutit Co.). The resin, after grinding, was sieved and 700 g. of the 60–80-mesh material was activated with HCl as described by Partridge & Westall (1949). The Zeo-Karb was poured into a column 5·8 cm. wide by 91·4 cm. long as an aqueous suspension and allowed to settle. After all the resin had been added, distilled water was forced up the column from the bottom so that the particles were freely suspended; the water flow was then reversed and the particles allowed to fall into position. By this means an evenly packed column is ensured. The column was washed with distilled water until the pH of the effluent had risen to 5·0–6·0.

The charcoal effluent (66 l.) was added to the column at such a rate that the outflow was approximately 5 l./hr. Ninhydrin tests were carried out on the effluent to check that the column was not being overloaded; the presence of a little glutamic acid in the effluent was ignored. When all the charcoal effluent had been added the amino-acids were displaced with 0·4 M-NH_3 solution added at a rate of approximately 1·5 l./hr. As soon as a significant amount of amino N appeared in the effluent it was collected in 20 ml. fractions by means of a fraction collector. A sample from every fifth tube was run on a one-dimensional paper chromatogram in butanol-water-acetic acid solvent (4:5:1) (Partridge, 1948) according to the method of Consden, Gordon & Martin (1944) in order that the amino-acid composition of the effluent might be followed. The elution with NH_3 was continued until the amount of N in the effluent became insignificant. The contents of the tubes were bulked into ten fractions according to their amino-acid composition. These were concentrated by evaporation under reduced pressure and N determinations (Kjeldahl) carried out. The distribution of N in a typical column effluent is shown in Table 3. Each of the last six fractions, in which proline was either absent or only present in very low concentration, was further concentrated and any crystalline material removed. The various fractions were then submitted to biological tests, the results of which are also shown in Table 3. It will be seen that although the activity was present in all of the last six fractions, it was concentrated in one fraction (*F*). The distribution of activity in the combined fractions from all three Zeo-Karb columns is shown in Table 1. It will be seen that for the most active fraction the toxic dose contained only 56 mg. N.

Before the Zeo-Karb was used again it was treated with 5% (w/v) NaOH solution at 55–60° to remove any aromatic amino-acids which may have been adsorbed and was then regenerated with 5 N-HCl (Newkirk & Handelman, 1949).

Fractionation of effluent from Zeo-Karb 215 on a paper column

The use of a column consisting of powdered paper was originally suggested by Consden *et al.* (1944). In the present case a powdered paper known commercially as solka floc (200-mesh grade; obtainable from Johnsen, Jorgensen and Wettre, 26 Farringdon Street, London, E.C. 4) has been used. The paper was washed thoroughly with distilled water, boiled for 30 min. with 5% acetic acid, washed with water, boiled with absolute ethanol, filtered, washed with ether, and dried in an oven at 110°. Approximately 1 kg. of the dried paper was then suspended in about 6 l. of acetone and loaded into a glass column 120 cm. long by 6·5 cm. diameter. The paper suspension was added in a continuous stream and the acetone allowed to flow freely from the column. After all the suspension had been added the column was washed with a further 6 l. of acetone. The level of the acetone was allowed to fall until it was just above the top of the paper and a solvent consisting of a mixture of butanol, water and acetic acid added. The composition of this solvent was similar to the 4:5:1 mixture described by Partridge (1948), but it was more economically prepared by mixing *n*-butanol 63%, water 27% and acetic acid 10% by volume together and allowing it to stand for 48 hr. before use. During this time esterification takes place and equilibrium is established; if the solvent separated into two layers then only the upper layer was used. The solvent (15 l.) was passed through the column at as fast a rate as possible until the paper had become saturated with water. This could be checked by adding a drop of water to 10 ml. samples of the effluent, since, when the solvent is saturated with water, a cloudy solution is produced under these conditions. The solvent level was then reduced to the level of the paper and the column was ready for fractionation.

Only the most active fractions (*F*, Table 1; 194–205, Table 3) from the Zeo-Karb column were fractionated on the paper column, the less active fractions being combined and first refractionated on a further Zeo-Karb 215 column. The solution of amino-acids to be fractionated (300 mg. N) was evaporated to dryness *in vacuo*, a little ethanol added and the evaporation repeated twice. The residue was dissolved in about 300 ml. of the butanol-acetic acid solvent to which 6 ml. of glacial acetic acid and 10 ml. of ethanol had been added. This usually resulted in a quite clear solution, but in the event of the solution being cloudy it was first cleared by centrifugation and only the supernatant solution used for fractionation. The clear solution was then added to the

Table 3. *Fractionation on Zeo-Karb* 215

Fraction tubes no.	N from column (mg.)	N after removal of crystalline material (mg.)	No. of toxic doses	N/toxic dose (mg.)
76–123	1930			
124–159	1700	—	Not toxic	Not toxic
160–171	547			
172–188	700			
189–193	216	162	1	162
194–205	618	375	8	47
206–213	245	173	1·5	115
214–220	212			
221–239	174	183	1	183
240–261	18			
Total N in mg.	6360	893	—	—

column without disturbing the surface. This solution was allowed to percolate into the paper before the developing solvent was added. The flow rate was adjusted to 1 drop/sec. and the effluent was collected in 18 ml. fractions on a fraction collector, approximately eighty fractions being collected in 24 hr. Samples from every fifth tube were run on single-dimensional paper chromatograms (Consden *et al.* 1944) in butanol-acetic acid in order to determine the amino-acid composition of the effluent. The fractions were then grouped according to the composition of their ninhydrin-positive substances as shown in Table 4. All the groups were biologically tested, but only one (*G*, Table 1, 355–475, Table 4) possessed any activity; a typical fit being produced by material containing as little as 4 mg. N. The ninhydrin-reacting substance in (*G*) gave initially a yellow colour with ninhydrin, but on heating the colour changed through brown to the usual purple.

Table 4. *Fractionation on paper column*

(300 mg. N on 1 kg. paper. Each tube contained 18 ml. of solvent.)

Tube no.	Total N (mg.)	Amino-acid composition
150–180	68	Leucine + trace valine
181–209	53	Valine + trace leucine
210–229	22	Unidentified, purple ninhydrin
230–255	56	Proline
256–269	18	γ-Aminobutyric acid
270–282	6·5	Traces of various unidentified ninhydrin positive substances
283–304	25	
305–315	8·2	
316–340	14·6	
341–354	5·6	
355–475	16	Toxic substance
476–		Histidine positive Pauly reaction

When the amount of ninhydrin-positive material in the fractions fell to an insignificant amount the solvent was changed to an 85% ethanol-water mixture in order to remove the last traces of substances with a very low R_F. 2 l. of 85% (v/v) ethanol followed by 6 l. of 50% (v/v) ethanol were added and the effluent collected. After concentration under reduced pressure these fractions were tested biologically, but failed to give any positive reaction. The solvents were then used in the reverse order. After the addition of 2 l. of butanol-acetic acid solvent the column was again ready for further fractionations.

Treatment of the active fraction from the paper column

The fractions (*G*, Table 1; 355–475, Table 4) from the paper column were pooled and evaporated to dryness under reduced pressure. The acetic acid was removed by the addition of successive small quantities of distilled water and evaporation to dryness. The residue was dissolved in the minimum amount of distilled water and any insoluble material was filtered off. After the addition of a little absolute ethanol to the solution the toxic substance crystallized in the form of small colourless beads. The toxic dose of this crystalline material, m.p. 226–232° (decomp.), when fed to a ferret over a period of 5 days was 3 mg. of solid. The total number of toxic doses obtained from 17·7 kg. of flour was about 12, giving an overall yield of 8·5%. A phase-contrast photograph of the recrystallized material is shown in Fig. 1.

Alternative methods of isolation

While this work was in progress, Bentley, McDermott, Pace, Whitehead & Moran (1949*a*) reported the purification of a toxic substance from agenized zein. In many respects the methods used by these workers corresponded to those employed in the present work. It seemed possible that some of their methods might also prove useful in the extraction of a toxic substance from flour.

Butanol extraction. A sample of an active fraction from a Zeo-Karb 215 column equivalent to 380 mg. N (two toxic doses) was made up to 50 ml. with distilled water and extracted with *n*-butanol at 60° for 22 hr. as described by Dakin (1918). On examination of the two extracts by paper-partition chromatography in butanol-acetic acid-water it was found that the butanol fraction contained most of the amino-acids (357 mg. N). The water fraction was completely lacking in valine and leucine and contained 21·5 mg. N. Biological tests showed that both fractions were of approximately equal activity. Although this method was attractive in that the total N for one toxic dose was reduced from 190 to 21·5 mg. N the loss of 50% of the toxic material rendered the process uneconomic. The appearance of 50% of the activity in the butanol extract was contrary to the experience of Bentley *et al.* (1949*a*). This discrepancy must now be attributed to some small difference in technique as it appears that the toxic substance from wheat flour is identical with that from zein.

Partition between phenol and acid. A sample of the concentrated toxic factor from Zeo-Karb 215 was partitioned between equal volumes of phenol and 0·1 N-HCl. The amino-acids were distributed almost equally between the two layers and the method was not considered useful in the present case.

Degradation of crystalline toxic substance

As the quantity of crystalline material available after biological testing was about 10 mg. it was not possible to isolate and characterize degradation products. When the identity of a degradation product had been indicated by chromatography on paper it was mixed with an authentic specimen and an attempt made to separate the substances by partition chromatography in two dimensions on paper (phenol/NH_3 followed by acetic acid/butanol/water). If no separation was achieved the unknown was assumed to be identical with the known specimen.

With this reservation, the following substances were identified as the products of acid hydrolysis (6 N-HCl for 24 hr.) followed by treatment with H_2O_2; homocysteic acid, methionine sulphoxide, methionine sulphone, α-aminobutyric acid and homoserine. In addition, a small quantity of undegraded toxic substance was identified. The relative positions of these substances on a chromatogram is shown in Fig. 2.

A sample (2 mg.) of crystalline toxic substance was desulphurized by treatment with Raney nickel by the method of Fonken & Mozingo (1947). After removal of the solid nickel a small quantity of NiS was precipitated when H_2S was passed into the filtrate. After removal of NiS the filtrate was divided into four portions. One quarter was chromatographed on paper using collidine/lutidine as solvent. The R_F indicated that the degradation product was an aminobutyric acid. One quarter was now mixed with synthetic α-aminobutyric acid and one quarter with γ-aminobutyric acid. Each mixture was chromatographed on paper. The

mixture of unknown substance and γ-aminobutyric acid separated into two ninhydrin-positive spots; the mixture of unknown and α-aminobutyric acid could not be separated. Methionine gave α-aminobutyric acid under similar conditions.

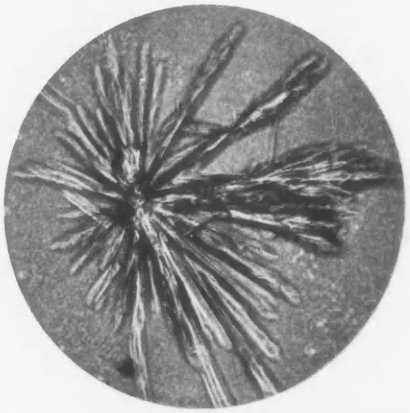

Fig. 1. Phase-contrast photograph of the crystalline toxic substance from agenized wheat flour (×180).

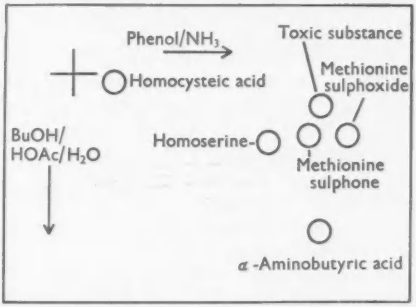

Fig. 2. Diagrammatic representation of the position of the toxic substance and its degradation products (acid hydrolysis) in a two-dimensional chromatogram.

Treatment of some methionine peptides with NCl_3

The following peptides were treated with NCl_3 in $CHCl_3$: methionylglycine, carbobenzyloxymethionylmethionine amide, methionylmethionine, carbobenzyloxymethionylmethionine, carbobenzyloxy-L-methionine amide, carbobenzyloxyglycyl-L-methionine and carbobenzyloxy-L-methionylglycine. A sample (20 mg.) of each was suspended in approximately 10–15 ml. of $CHCl_3$. Air containing NCl_3 from approximately 50 ml. of Cl_2 was passed into each. The solutions were left at room temperature for about 1 hr. when the remaining NCl_3 was removed by sucking a vigorous stream of air through the solutions until no further $CHCl_3$ remained. Each specimen was dissolved in 6N-HCl (0·2–0·3 ml.) and heated in a sealed tube for 11 hr. at 100–105°. The amino-acid composition of each hydrolysate was studied by two-dimensional paper-partition chromatography using butanol acetic acid and phenol/NH_3 as solvents. Wherever a component was detected which resembled the toxic factor in R_F values a second chromatogram was run with a mixture of peptide hydrolysate and crystalline toxic substance. In every case it was possible to separate the toxic substance from all the peptide degradation products and it was, therefore, concluded that in no case had the NCl_3 treatment of a peptide given rise to the toxic substance.

RESULTS AND DISCUSSION

Isolation

In order to isolate the toxic substance from wheat flour it was obviously desirable to obtain a flour possessing the maximum activity. As a result of many experiments it was concluded that it was not practicable to treat wheat flour with more than about ten times the amount of nitrogen trichloride which was normally used commercially. In the later stages of the work commercial gluten itself was treated with nitrogen trichloride, since the toxic substance is associated with the gluten fraction of the flour, but the product was only slightly more active than that obtained when the flour itself was treated with nitrogen trichloride. The digestion of agenized gluten with pepsin and trypsin resulted in a very considerable reduction of the average molecular weight, for about 80% of the nitrogen was dialysable at this stage. Since on a nitrogen basis the non-dialysable material was as toxic as the dialysable, it was apparent that the toxic substance was an integral part of the protein and was not a low-molecular-weight impurity.

Although it is possible that the non-dialysable residue from the enzymic digestion could have been satisfactorily hydrolysed by acid, it was felt that the elimination of possibly 'resistant' non-dialysable peptides at this stage was an advantage.

The acid hydrolysis caused a destruction of about 30% of the toxic substance (see Table 1), but the proportion of amino nitrogen to total nitrogen was raised from 25% to approximately 60%; thus a further substantial reduction in the average molecular weight was achieved. It was felt that fractionation would be so simplified by this reduction in molecular weight as to make an acid hydrolysis an essential step in spite of the ensuing loss of activity.

Electrodialysis achieved a very considerable purification of the toxic substance with negligible loss. Table 1 shows that the toxic dose contained 2300 mg. nitrogen before electrodialysis and 500 mg. nitrogen after electrodialysis and removal of crystalline material from the neutral dialysate. About 36% of the total amino-acids in hydrolysed gluten consist of glutamic acid. Thus most of the nitrogen in the anode compartment was glutamic acid. The basic amino-acids, arginine, lysine and

histidine, together account for about 10% of the total solids and, of course, a very much higher percentage of the N. Thus it is not surprising that even though only a crude fractionation was achieved by this process a great reduction in the ratio nitrogen/toxic dose was achieved.

The removal of aromatic amino-acids by charcoal was found to be a necessary step before fractionation of the mixture on a Zeo-Karb column, since we, like Partridge (1949), found that in the presence of aromatic amino-acids fractionation on Zeo-Karb 215 is adversely affected.

The toxic substance was found in the fractions from the Zeo-Karb column immediately following proline. Although activity was spread over rather a large number of fractions, the maximum activity appeared in a relatively small fraction. Only this fraction was used for the next step in purification and the other active fractions were combined and refractionated on Zeo-Karb.

Single-dimension paper chromatography of the various active fractions from Zeo-Karb indicated the presence of at least two major abnormal components. One of these, eventually identified as the toxic substance, had R_F in butanol/acetic acid of 0·07 and in phenol/ammonia of 0·64. The second abnormal component was provisionally identified, in the first instance by paper chromatography, as γ-aminobutyric acid; it was then isolated, analysed and found indistinguishable from an authentic specimen of γ-aminobutyric acid. A yield of 46·7 mg. of thrice recrystallized γ-aminobutyric acid, m.p. 193°, was obtained from 1800 g. of gluten.

Since it has not been possible to isolate γ-aminobutyric acid from unagenized gluten by the methods which were successful in the case of agenized gluten, it must be concluded that γ-aminobutyric acid arises as a result of the action of nitrogen trichloride on the protein. Since all fractions from the paper column other than the one containing the toxic substance were biologically inactive, it is concluded that the toxicity of agenized wheat flour cannot be attributed in any way to the presence of γ-aminobutyric acid. Further, Keil (1932) did not detect any toxic action arising from the subcutaneous injection of the sodium salt of γ-aminobutyric acid to human subjects.

The most active fraction from Zeo-Karb was still a complex mixture, but a considerable quantity of neutral amino-acid was removed by concentration and crystallization. The activity remained entirely in the mother liquors and these were found, by two-dimension paper chromatography, to contain at least sixteen ninhydrin-positive substances.

Fractionation on a column of powdered paper was a most efficient method. The degree of fractionation depended on the amount of material put on the column. When a column containing 1 kg. of paper was loaded with a mixture containing 100 mg. nitrogen, fractionation was complete and every substance appeared in the effluent unmixed with substances of similar R_F values. When the load was increased to 300 mg. nitrogen the substances with high R_F values were not completely separated; when the load was increased to 500 mg. nitrogen even the components with low R_F values were mixed. The progress of fractionation of a 300 mg. nitrogen batch on a paper column is shown in Table 4.

In assessing the value of a paper column as a method of fractionation of a particular amino-acid mixture, it was of great convenience to find that a column and a paper strip behaved in a similar fashion. There were, however, small but significant differences, thus proline appeared in the effluent from the column before γ-aminobutyric acid, whereas on a paper strip, in the same solvent mixture, the order was reversed. The loss of about 30% of the toxic substance on the paper column cannot be explained.

Some difficulty was experienced in the crystallization of the toxic substance from the paper column. There was contamination with a small quantity of carbohydrate, presumably the product of slow decomposition of the very large quantity of paper in contact with acetic acid. Similar difficulties have been reported by Moore & Stein (1949) when using starch columns with acidic solvents.

Degradation and comparison with other toxic factors

With the reservation made in the appropriate experimental section, the identification of α-aminobutyric acid as a product of desulphurization with nickel suggested that the toxic substance was derived from methionine. The production of methionine sulphoxide and methionine sulphone on acid hydrolysis supported this view. The occurrence of homoserine, α-aminobutyric acid and homocysteine as products of acid hydrolysis suggested in the first instance that we were dealing with a peptide, but the failure to identify any product of desulphurization other than α-aminobutyric acid was contrary to this conclusion.

In the earlier stages of this investigation we were of the opinion that the toxic substance was a peptide and we believed that our substance differed from that which was produced from zein by treatment with nitrogen trichloride. This belief was occasioned by the suggestion of Bentley *et al.* (1949*a*) that a toxic factor which they had obtained in a highly concentrated form from zein did not give a reaction with ninhydrin. However, when Bentley *et al.* (1949*b*) crystallized their toxic substance from zein and degraded it to α-aminobutyric acid by hydrogenolysis (Bentley, McDermott, Pace, Whitehead & Moran, 1950) it became obvious to us that the toxic substance which we had isolated from wheat flour

was closely similar to and probably identical with that already isolated from zein. Identity was confirmed when Dr L. Reiner supplied us with a sample of the crystalline substance isolated from zein in a parallel, but independent investigation (Reiner, Misani, Fair, Weiss & Cordasco, 1950). Dr Reiner's material could not be separated from our own by two-dimension partition chromatography on paper, and on acid hydrolysis it gave rise to the same five ninhydrin-positive substances.

We have been unable, through lack of sufficient material, to obtain a full elementary analysis of our material; an analysis has, however, been published by Bentley *et al.* (1950) and the deduction has been drawn by these authors that the molecule $C_5H_{12}O_3N_2S$ can be regarded as being derived from methionine sulphoxide by addition of NH or from methionine sulphone by replacement of O by NH. All the hydrolytic degradation products which we have 'identified' by chromatography could be envisaged as possible degradation products of such a structure.

It seemed reasonable to expect that treatment of a single methionine peptide with nitrogen trichloride would give rise to the toxic substance, but, as indicated in the experimental section, we were unable to detect any formation of the toxic substance in the reaction between nitrogen trichloride and six different methionine peptides. It may be that the chromatographic method used to detect formation of the toxic substance was insufficiently sensitive and that a very low yield of toxic substance was obtained; it seems more probable, however, that the reaction conditions were not analogous to those which exist in the agenization of flour.

Although it has not been possible to produce typical epileptiform fits in all animals by feeding agenized flour, it cannot be assumed that the toxic factor is harmless in those animals which show no nervous response. In view of the very serious and eventually fatal outcome of the treatment of dogs, ferrets, rabbits and cats with the toxic substance, the use of nitrogen trichloride as an ageing accelerator in the manufacture of flour seems highly undesirable.

SUMMARY

1. The isolation of a toxic substance from agenized wheat flour is described.

2. The probable identity of this substance with a substance having similar physiological properties from agenized zein is indicated.

3. The toxic substance was isolated from an acid hydrolysate of the gluten fraction of flour and is regarded as part of a flour protein.

4. Large-scale partition chromatography on paper-pulp columns was developed as a method of fractionation of complex mixtures.

5. Degradation experiments suggested that the toxic substance was a derivative of methionine. The probable structure is given.

6. Methionine peptides treated with nitrogen trichloride did not give rise to the toxic substance.

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REFERENCES

Baker, J. C. (1921). U.S. Patent, 1,367,530.
Bentley, H. R., McDermott, E. E., Pace, J., Whitehead, J. K. & Moran, T. (1949*a*). *Nature, Lond.*, **163**, 675.
Bentley, H. R., McDermott, E. E., Pace, J., Whitehead, J. K. & Moran, T. (1949*b*). *Nature, Lond.*, **164**, 438.
Bentley, H. R., McDermott, E. E., Pace, J., Whitehead, J. K. & Moran, T. (1950). *Nature, Lond.*, **165**, 150.
Blish, M. J. (1945). *Advanc. prot. Chem.* **2**, 337.
Campbell, P. N., Work, T. S. & Mellanby, E. (1950). *Nature, Lond.*, **165**, 345.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.
Cox, G. J., King, H. & Berg, C. P. (1929). *J. biol. Chem.* **81**, 755.
Dakin, H. D. (1918). *Biochem. J.* **12**, 290.
Fonken, G. S. & Mozingo, R. (1947). *J. Amer. chem. Soc.* **69**, 1212.
Keil, W. (1932). *Hoppe-Seyl. Z.* **207**, 248.
Mellanby, E. (1946). *Brit. med. J.* **2**, 885.
Mellanby, E. (1947). *Brit. med. J.* **2**, 288.
Melnick, D. & Cowgill, G. R. (1937). *J. Nutrit.* **14**, 401.
Moore, S. & Stein, W. H. (1949). *J. biol. Chem.* **178**, 53.
Moran, T. (1947). *Lancet*, **2**, 289.
Newell, G. W., Erickson, T. C., Gilson, W. E., Gershoff, S. N. & Elvehjem, C. A. (1947). *J. Amer. med. Ass.* **135**, 760.
Newkirk, T. H. & Handelman, M. (1949). *Industr. Engng Chem.* **41**, 452.
Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
Partridge, S. M. (1949). *Biochem. J.* **44**, 521.
Partridge, S. M. & Westall, R. G. (1949). *Biochem. J.* **44**, 418.
Radomski, J. L., Woodard, G. & Lehman, A. J. (1948). *J. Nutrit.* **36**, 15.
Reiner, L., Misani, F., Fair, T. W., Weiss, P. & Cordasco, M. G. (1950). *J. Amer. chem. Soc.* **72**, 2297.
Schramm, G. & Primosigh, J. (1943). *Ber. dtsch. chem. Ges.* **76** B, 373.
Svensson, H. (1948). *Advanc. prot. Chem.* **4**, 251.
Tiselius, A., Drake, B. & Hagdahl, L. (1947). *Experientia*, **3**, 21.
Wagner, J. R. & Elvehjem, C. A. (1944). *J. Nutrit.* **28**, 431.

The Carbohydrates of the Jerusalem Artichoke and other Compositae

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The most fully investigated polysaccharide of the Compositae is inulin (named from *Inula helenium*), first obtained from the tubers of the Jerusalem artichoke, *Helianthus tuberosus* L. (Rose, 1804), but more easily prepared from the dahlia tuber (cf. McDonald, 1946). ('Artichoke' is used to indicate Jerusalem artichoke throughout this paper.) It has long been evident (cf. Tanret, 1893*a*, *b*) that inulin may constitute only a small fraction of the total carbohydrate present in the artichoke tuber, the remainder resembling inulin in being composed essentially of fructofuranose units, but having a greater solubility in water and in aqueous ethanol. The term 'inulide' has been used by French workers (Wolff & Geslin, 1918) to describe substances of this type, which occur also in the tissues of many other Compositae (cf. Colin, 1919). Lemoigne (1941*a*, 1942) has published a comprehensive review of existing knowledge of the carbohydrates of the artichoke.

In the course of an investigation into the transformations of fructose polysaccharides in the tubers we have made a survey of the carbohydrates present, using new methods, in particular paper partition chromatography. Although it was not our main intention to investigate the composition of the tubers, and some of the techniques employed are perhaps not the most suitable for that purpose, it is clear from the results presented here that existing ideas about the tuber carbohydrates must be modified. A preliminary account of this work has been given (Bacon & Edelman, 1949).

METHODS

Standard solutions of sugars. Glucose and fructose were dissolved in saturated benzoic acid solution (Folin, 1929), sucrose in water, and the concentration checked by polarimetry. No glucose could be detected by glucose oxidase in the fructose sample used.

Polarimetry. The optical rotations of solutions were read in 2 dm. tubes using a sodium lamp as light source.

Dry weight determinations. Tubers were cut out into quarters and thin slices taken from the cut surfaces. Samples with a fresh weight of 8–12 g. were dried for 24 hr. at 100°; a further 24 hr. drying decreased the weight by only 10–20 mg.

Deproteinization. Routinely, extracts were deproteinized by adding 0·1 vol. of 30% (w/v) lead acetate ($Pb(C_2H_3O_2)_2.3H_2O$) solution. To the filtrate was added 3% (w/v) sodium oxalate solution in the proportion 2·5 : 16 ml. of filtrate. Occasionally minimal amounts of each reagent were used.

Estimation of total reducing substances (TRS). The method used was essentially that of Miller & Van Slyke (1936) with slight modifications (cf. Bacon & Bell, 1948). Reducing sugar in easily hydrolysed combination was determined by TRS estimations before and after hydrolysis, which was done by adding 0·1 vol. of 5·5% (w/v) oxalic acid solution to the sample and heating the mixture for 30 min. in a boiling-water bath. The concentration of carbohydrate was so chosen that a dilution of 20 times or more was needed before the TRS estimation; under these conditions neutralization of the acid was unnecessary. Contrary to the previous experience of one of us (J. S. D. B.; see Bacon & Bell, 1948) we found that fructose gave 1·06 times the reduction by an equivalent amount of glucose. The correction required was usually about the same as the experimental error because the ratio of fructose to glucose in the whole extracts was approximately 4 : 1. The TRS is expressed as hexose; the standard used is stated where the discrepancy between glucose and fructose is important.

Estimation of glucose by glucose oxidase. Aspergillus niger (National Collection of Type Cultures, No. 594), subcultured on malt-agar slopes, was inoculated into flasks containing a liquid culture medium (0·5% $NaNO_3$, 0·1% $MgSO_4.7H_2O$, 0·3% K_2HPO_4, 10% glucose (w/v)) and the flasks incubated at 30°. The appearance of the enzyme was followed by measuring the O_2 uptake of small portions of the medium in the presence of glucose.

The liquid was poured off, dialysed against running tap water and filtered through a folded filter paper (J. Barcham Green Ltd., Hayle Mill, Maidstone, no. 904½). The dialysed material was neutralized with NaOH and concentrated *in vacuo* in a water bath at 35–40°. The product was a clear yellow liquid which contained a little invertase activity in addition to the glucose oxidase, indicated by a slow O_2 uptake in the presence of sucrose. Glucose was estimated in 0·2 M-acetate buffer (pH 5) either manometrically (Keilin & Hartree, 1948) or by difference in TRS before and after incubation with the enzyme.

Estimation of ketose. This was done colorimetrically using the method of Cole (unpublished; cf. Bacon & Bell, 1948) with a fructose standard. Chromatographic evidence suggested that fructose was the only ketose present in the extracts. The method gave the expected amount of fructose when applied to sucrose; it gave the same ketose value with the tuber extracts before and after acid hydrolysis, but this cannot be taken as evidence that it measures accurately fructose combined as in inulin.

Paper partition chromatography

The apparatus used was similar to that of Consden, Gordon & Martin (1944) and Partridge (1948). Strips of sponge rubber were clipped round the tops of the drainpipes, and a sheet of plate glass resting on this gave a satisfactory air-tight seal. The pipes stood in lead or aluminium trays con-

taining the aqueous phase. Large sheets of paper were run in wooden tanks lined with lead (for phenol and collidine). Butanol-acetic acid mixtures rapidly corroded the lead, and an aluminium tank lagged with cotton wool was used for this solvent. The troughs were constructed of stainless steel. Strips (15 × 60 cm.) and sheets (60 × 60 cm. or 57 × 46 cm.) of Whatman no. 1 filter paper were used. The solvent was removed from the paper by heating it in a current of air in a specially constructed oven.

Solvents. In addition to the solvent mixtures described by Partridge (1948), *iso*propanol-aqueous NH_3 (9 : 1, v/v), *n*-butanol saturated with N-aqueous NH_3 and mixtures of varying proportions of *n*-butanol-acetic acid-water were used. Of all mixtures investigated, that composed of 40 ml. *n*-butanol, 10 ml. acetic acid and 50 ml. water (called hereinafter 'butanol-acetic') (Partridge, 1948) was found to give the most satisfactory results with the tuber carbohydrates, and was used for most of the qualitative and all the quantitative chromatography.

Reagents for detection of carbohydrates. The developed and dried chromatograms were sprayed with the appropriate reagents and heated in the oven. (i) *Phloroglucinol* (Horrocks & Manning, 1949). Spots containing free or combined ketose show pink when heated at 100–110° with this reagent. Glucose is not detected. (ii) *Naphthoresorcinol* (Partridge, 1948) and similar reagents made with resorcinol and orcinol were tried. Resorcinol gave a delayed colour production during a day at room temperature following the usual heating in the oven; orcinol gave green spots on a pink background. (iii) *Benzidine* (Horrocks & Manning, 1949). Spots containing free aldose groups show a chocolate brown colour when heated at 100–120°, but glucose in easily hydrolysed combination gives a delayed colour production after several days at room temperature. Fructose, either free or combined, is detected in relatively large quantities only (about 100 μg. or more). (iv) *Benzidine-trichloroacetic acid.* 0·5 g. Benzidine (A.R.) in 10 ml. acetic acid, 10 ml. 40% (w/v) aqueous trichloroacetic acid, 80 ml. ethanol. This was found to be the most useful reagent for the materials studied. It is very sensitive to both free and combined glucose, giving a more intense brown colour with this sugar than the benzidine reagent described above, and will detect less than 1 μg. A yellow colour is developed with free or combined fructose, but the reagent is appreciably less sensitive to this sugar. (v) *Aniline acid phthalate* (Partridge, 1949).

Treatment with invertase on the paper. Sugars which are attacked by invertase can be treated with this enzyme on the paper. Chromatograms run in one direction in butanol-acetic were sprayed with a 4% (v/v) dilution of 'invertase concentrate' (British Drug Houses Ltd.) in water and hung in the oven at 50° over a free water surface for 15–30 min. The products of hydrolysis were separated in the usual way by running the chromatogram at right angles to the original direction. This treatment hydrolysed completely up to 200 μg. of sucrose and did not interfere with R_F values or colour development.

Photography of chromatograms. After colour development the chromatograms were photographed: a sheet of Kodak Reflex Contact Document paper was placed face downwards in close contact with the chromatogram, the two being held between pieces of plate glass, and illuminated by a 40 W. electric lamp 35 cm. above the chromatogram. With exposures ranging from 20 to 40 sec. satisfactory negatives were obtained from which positives (cf. Fig. 2) were made by transmitted light, often showing better contrast than the original chromatogram. The procedure was used routinely because the background colour of the chromatograms intensified with keeping, and made faint spots almost invisible, particularly with the phloroglucinol reagent.

Quantitative estimation. Quantities (5 μl.) of the solution to be analysed were put on to strips or sheets of filter paper using an 'Agla' micrometer syringe (Burroughs Wellcome and Co.); the measurement has an accuracy of ±1%. The paper was run for a suitable time (usually 2 days) in butanol-acetic and dried at 60–80°. Every second or third strip, each corresponding to one application of the solution, was cut out and sprayed with an appropriate reagent. The positions of the spots in the unsprayed strips were then deduced and rectangles from sufficient strips (usually two to four) to give enough material to estimate were combined for a single estimation. The carbohydrate was extracted by heating the paper to 75–80° for 20 min. in a known volume of water in a glass-stoppered tube, and shaking the tube vigorously to disintegrate the paper. The resulting suspension was filtered. The estimation was carried out on a sample of the filtrate. This procedure was found satisfactory for ketose estimation, as blanks on the paper gave density readings of less than 0·01 in nearly every case; recoveries of 95–100% were obtained with sucrose, but lower recoveries, namely 85–95%, were found with tuber extracts. Estimation of TRS was not as accurate or convenient. The estimation is less accurate than that used for fructose for amounts of material less than 80 μg., so more material had to be taken. Blanks were of the order of 10 μg./spot, varying with the area of paper, and were not eliminated by washing the paper with cold water before application of the solution to be estimated. Combined reducing sugar was hydrolysed after extraction from the paper by adding 0·5 ml. 0·5 N-HCl to 4·0 ml. of the filtrate contained in the tube in which the estimation was to be carried out, and heating it in a boiling-water bath for 30 min. The tubes were cooled in water and the acid neutralized by adding 0·5 ml. 0·5 N-NaOH, after which alkaline ferricyanide was added and the estimation completed by the normal procedure.

RESULTS

Carbohydrates of the artichoke tubers

Sources of material. At first, small lots of tubers were purchased as they were required, during November 1948, but later a sackful was bought and stored in a moderately cool cellar (7–8°). Although bought at different times these tubers were probably all from the same batch, variety unknown. During 1949 a crop was raised from some of these tubers, which were planted in a sunny position on the outskirts of Sheffield. The plants grew 3–3·5 m. high, and although flower buds formed they did not open. A few tubers were taken out of the ground in mid-September, when they were still very small, but the majority were left there until April 1950 when the bulk were harvested and some small tubers replanted. In the autumn of 1949 another sackful of tubers, indistinguishable from the previous year's batch, was purchased; these were named 'Bedfordshire Pinks' and had probably been grown in the Midlands.

Extraction of tubers. The tubers were thoroughly scrubbed in running cold water, cut into pieces and disintegrated in a Waring blender with about half their weight of water. The proportion of water required depended to some extent on the losses of water which the tubers had suffered in storage. When about 10 % water had been lost the tubers became rubbery in consistency and disproportionately large amounts of extra water were needed to get satisfactory blending. The dry weight of tubers was approximately 20 % of the fresh weight (see Table 1). During the blending, and afterwards, the mash darkened, presumably through the action of phenol oxidases, but this could be largely prevented either by adding potassium cyanide to a final concentration of about 0·005 M, or by removing the outer layers of the tubers. A combination of these two procedures gave almost colourless preparations. The mash obtained was squeezed through gauze or madapollam, yielding an opalescent liquid. A few extracts, which are mentioned below, were made in other ways.

Examination of extracts

pH. The pH of all extracts was about 6·5, irrespective of the maturity and age of the tubers. The addition of calcium carbonate to the tubers during the extraction process to protect the carbohydrates from hydrolysis was therefore not necessary. Its presence when the mash was boiled with water seemed in our experience to be a disadvantage, since it encouraged the extraction of gelatinous material insoluble in 50 % (v/v) aqueous ethanol.

Deproteinization. When these experiments were begun, the use of lead acetate and sodium oxalate was thought sufficient both to remove protein and to destroy any enzymic activity, but it was discovered that the optical rotation of fresh extracts cleared in this way fell slowly when they were kept at room temperature; simultaneously the free TRS increased. If the extracts were boiled before deproteinization they showed no such change in rotation. The change could not be ascribed to a reaction between cyanide and free reducing sugar in the extracts; a 2 % (w/v) solution of fructose in 0·005 M-potassium cyanide showed no change in rotation when kept for 2 days at room temperature. It seems certain that a hydrolytic enzyme system was responsible, because later experiments (Edelman & Bacon, 1950) demonstrated that the hydrolytic system in the tubers was relatively stable in the presence of lead acetate. All extracts to be used for analyses were accordingly heated in a boiling-water bath for 10 min. as soon as possible after their preparation, usually reaching 80° within 10–15 min. of the beginning of blending.

Optical rotation. The rotations of deproteinized extracts varied considerably; Table 1 shows typical results, with details of the maturity and age of the tubers.

Table 1. *Composition and optical rotation of tuber extracts*

(All extractions were carried out as described under Methods, with the exception of that dated 8 Feb. 1949 which will be described later (Edelman & Bacon, 1950). Where dry weight determinations had not been made, the calculations of g. TRS after mild hydrolysis/100 g. fresh tuber were made assuming 80 % water.)

Date bought	Date dug up	Date extracted	Dry matter (g./100 g. fresh tuber)	Total reducing substances (TRS) after mild acid hydrolysis (g./100 g. fresh tuber)	Ketose as percentage of TRS after hydrolysis	Optical rotation corrected to undiluted juice (2 dm. tube)	$[\alpha]_D$ calculated on TRS after hydrolysis
8 Nov. 1948	—	17 Nov. 1948	—	15·5	—	−4·03°*	−10·4°*
22 Nov. 1948	—	23 Nov. 1948	—	—	—	−0·62°	—
1 Dec. 1948	—	1 Dec. 1948	—	15·7†	—	—	—
18 Dec. 1948	—	18 Dec. 1948	—	14·8	78	−0·78°	− 2·52°
3 Feb. 1949	—	3 Feb. 1949	—	—	—	+1·64°*	—
8 Feb. 1949	—	8 Feb. 1949	—	12·3	79	+1·59°	+ 5·15°
8 Feb. 1949	—	19 Mar. 1949	—	—	—	+3·28°	—
8 Feb. 1949	—	4 Apr. 1949	20·6	14·4	78	+0·13°	+ 0·38°
8 Feb. 1949	—	9 May 1949	20·3	14·2	78	+1·02°	+ 2·85°
—	28 Sept. 1949	6 Oct. 1949	18·4	14·4	91	−7·78°	−22·1°
—	28 Sept. 1949	7 Nov. 1949	21·1	16·0	86	−4·15°	−10·3°
—	28 Sept. 1949	24 Nov. 1949	25·0	19·4	85	−3·54°	− 6·84°
—	24 Nov. 1949	24 Nov. 1949	16·6	14·4	73	+1·02°	+ 2·94°
24 Nov. 1949	—	24 Nov. 1949	22·0	18·9	83	−3·87°	− 8·03°
—	5 Apr. 1950	1 May 1950	15·3	10·6	85	−0·36°	− 1·43°
—	5 Apr. 1950	1 May 1950‡	14·9	10·9	83	+0·05°	+ 0·19°

* These extracts were not boiled before deproteinization, but the rotations were read immediately afterwards.
† The $[\alpha]_D$ of the sugar liberated by mild acid hydrolysis was −64·8°.
‡ This extract was made by throwing diced tubers into boiling water and blending the whole when cold.

It is probable that some enzymic hydrolysis had taken place during extraction, causing a small change of rotation in the negative direction. Experiments in which the time of extraction in the Waring blender was varied indicated that differences of the order of $-0{\cdot}5°$ (calc. for undiluted juice) might be ascribed to this effect, which is thus small compared with the seasonal variations. Alternative methods of extraction, e.g. by first throwing diced tubers into boiling water, and later blending them, gave solutions with more positive rotations but otherwise very similar in composition (cf. the two extracts made 1 May 1950). It is by no means certain that the whole of the optical activity of extracts is due to carbohydrate.

On hydrolysis the rotation always became more negative, and the 'differential' $[\alpha]_D$ calculated from the change in rotation and the change in TRS for complete hydrolysis with oxalic acid at 60° was $-63{\cdot}5°$; that calculated for inulin ($[\alpha]_D$ $-40{\cdot}0°$) is $-53{\cdot}4°$, and for sucrose $-83{\cdot}0°$.

The $[\alpha]_D$ of the mixture of sugars produced by mild acid hydrolysis of one extract was $-64{\cdot}8°$, which corresponds to a mixture of fructose and glucose in the ratio 4·3 : 1. This calculation does not take into account the probable formation of difructose anhydrides during hydrolysis (Jackson & Goergen, 1929).

Spontaneous precipitates. Although 'inulin' is almost insoluble in cold water it can be extracted from plant tissues without the use of heat, and under these conditions it later appears as a dense white precipitate on the walls of the vessel holding the extract. No such precipitation was noticed with extracts made by the procedure described above during the period from November 1948 to the summer of 1949. An extract made by the method of Green (1888), i.e. by boiling diced tubers with water and concentrating the extract in an evaporating basin, deposited a small rather flocculent precipitate in the course of 2 months. The precipitate was centrifuged off, washed with water, and dissolved in water by warming to about 80°. On freezing, the material reprecipitated; it could be presumed to be inulin (ketose was present), but insufficient was available to characterize it further.

Material resembling inulin was obtained from extracts to which ethanol had been added: an equal volume of ethanol was added to fresh extract and the precipitate allowed to settle overnight before being filtered off. The clear filtrate, left for several months at room temperature, slowly deposited a translucent layer of carbohydrate on the walls of the flask. When an attempt was made to dissolve this material in a little cold water it did not dissolve completely, and the insoluble fraction proved to have the properties of inulin, i.e. it was a white, ketose-containing, substance, soluble in water at 80°, but slowly separating as a compact precipitate after the solution had been frozen and left in the refrigerator.

It would seem from these and similar experiments that 'inulin' was not present in significant amounts in extracts of the tubers obtained in November 1948. It is possible that the solubility of inulin in cold water (in itself not a very well defined property, cf. Lemoigne, 1941*b*) is increased by the presence of inulides, but in a qualitative experiment it was found that a commercial sample of inulin dissolved in tuber extract would still precipitate when the solution was frozen and kept cool.

Extracts made from the batch of tubers dug up in August 1949 showed a deposition of inulin almost at once; solutions prepared for polarimetry and left overnight had to be heated to dissolve the hazy precipitate which had formed even at room temperature. Fresh extracts showed a precipitation when frozen, but after having been heated to boiling point to destroy the hydrolytic enzyme system they showed a more delayed deposition of polysaccharide. It seems possible that the 'inulo-coagulase' effect of Wolff (1916) may be due to the addition of nuclei of aggregated polysaccharide to boiled extracts.

Extracts made from tubers grown under the same conditions, but dug up in a more mature condition several months later, showed a slight precipitation of inulin, but extracts made from the tubers harvested in April 1950 resembled those used in the winter of 1948–9 in giving no precipitate.

In the absence of any method of determining 'inulin' these observations may be taken to confirm earlier observations (Thaysen, Bakes & Green, 1929) that the amount of inulin in the tubers decreases during the autumn and winter, both relatively and absolutely.

Precipitation by ethanol. Although many experiments were carried out with a view to preparing polysaccharide by ethanol precipitation, to serve as a substrate for metabolic studies, it was clear that fractionation by this means was at best very unsatisfactory. Addition of ethanol to a concentration greater than 50 % (v/v) leads to the appearance of a heavy flocculent precipitate, but after this has been removed by filtration or centrifugation, precipitation continues for some months at room temperature. For example, of 23·4 g. of total ketose in an extract only 6·6 g. was precipitated by the addition of an equal volume of ethanol; but later the filtrate deposited a precipitate from which a substance resembling inulin was prepared (see above). This slow 'after precipitation' appears to be characteristic of the carbohydrates of the tuber (cf. discussion of the depolymerization of inulin, Lemoigne, 1941*c*), and would seem to make it impossible to define any fractions as being soluble or insoluble in a given concentration of ethanol (but see, nevertheless, Tanret (1893*a*, *b*), who also notes the considerable effects of temperature on the solubility of fractions).

Precipitation by acetone. The addition of 7–20 vol. acetone to extracts produced an immediate flocculent precipitate, and an opalescence which persisted for several days. Chromatographic analysis (see below) of the precipitates and supernatant fluids indicated that many substances are common to the two fractions.

Precipitation by aqueous baryta (cf. Tanret, 1893*a*). Aqueous baryta added in excess precipitated 15 and 2 %, respectively, of the total ketose of fresh extracts, and of the material soluble in 50 % (v/v) ethanol.

Free TRS. The free TRS of deproteinized extracts amounts usually to only 2–3 % of the total TRS after mild acid hydrolysis. Since reducing sugar is liberated from the carbohydrates by a hydrolytic enzyme system in the extracts (Edelman & Bacon, 1949) the exact value of the free TRS is probably not of much significance. Values have been found ranging from 0·25 to 0·75 g./100 g. fresh weight of tubers. In some extracts there has been chromatographic evidence for the presence of monosaccharides, but in others it seems that the bulk of the free TRS must either be non-carbohydrate in character, or alternatively due to reducing end groups on some of the polysaccharide present.

Combined TRS. The greater part, possibly all, of the combined reducing sugar is in a form liberated by mild acid hydrolysis. An experiment was carried out to discover whether more drastic acid hydrolysis would liberate further glucose in the extracts (see Table 2). From these results it seems that only an insignificant amount of the glucose can be present in combination with pyranosidic linkages, or with non-carbohydrate residues.

After mild acid hydrolysis the ketose content of the extracts is unchanged, and corresponds to 80–85 % of the TRS, the exact percentage depending on the maturity and age of the tubers (see Table 1). The remaining TRS liberated was accounted for as glucose by the use of glucose oxidase (see Tables 2 and 3).

It is evident from these data that the carbohydrates consist essentially of non-reducing saccharides, in which the majority of linkages are of the furanosidic type.

Paper partition chromatography

Qualitative. The extracts showed a series of spots, all detected with either phloroglucinol or benzidine-trichloroacetic sprays (see Fig. 1). Free hexoses, when

Fig. 1. Typical chromatogram of extract of artichoke tuber. Run in butanol-acetic for 3 days; sprayed with benzidine-trichloroacetic acid. The position of spot 1 corresponds with that of sucrose (see Fig. 3). (Second positive print.)

Table 2. *Comparison of effects of weak and more drastic acid hydrolysis on liberation of glucose from artichoke-tuber extract*

(2·0 ml. extract heated with 2·0 ml. of 10 N-H_2SO_4 in boiling-water bath for 2 hr. Cooled, and neutralized with 10 N-NaOH. Further 2·0 ml. heated with 0·4 ml. 5·5 % (w/v) oxalic acid in boiling-water bath for 30 min. Both hydrolysates diluted to 25 ml. with water. 2·0 ml. of each solution incubated at 40° in Warburg cups with 1·2 ml. 0·6 M-acetate buffer (pH 5), 0·3 ml. glucose oxidase and 0·1 ml. catalase solutions until O_2 uptake ceased. Values calculated per ml. original extract.)

	O_2 at N.T.P. (μl.)	Glucose calculated (mg.)	TRS (mg.)	Ketose (mg.)	Glucose (by difference) (mg.)
Weakly hydrolysed extract	1510	23·4*	97·5	74·5	22·6
More drastically hydrolysed extract	1520	23·6*	—	—	—

* 1 mg. glucose under the conditions of the experiment gave O_2 uptake of 64·5 μl.

Table 3. *Estimation of glucose in hydrolysed artichoke-tuber extract by glucose oxidase*

(1·0 ml. hydrolysed extract (diluted ×20) incubated at 40° in Warburg cup with 1·0 ml. 0·2M-acetate buffer (pH 5), 0·2 ml. glucose oxidase and 1 drop catalase (horse-kidney extract), until O_2 uptake ceased. Estimations done on contents of cup diluted to 50 ml. Values calculated per ml. of original extract.)

	O_2 uptake (μl. at N.T.P.)	Glucose calculated from O_2 uptake (mg.)	Ketose (mg.)	TRS (mg.)	Non-ketose (by difference) (mg.)	Glucose calculated as disappearance of TRS (mg.)
Untreated	—	—	79·5	101	21·5	—
Treated with glucose oxidase	1510	22·5*	80·5	78·2	—	22·8

* 1 mg. glucose under the conditions of the experiment gave O_2 uptake of 67·5 μl.

Table 4. *Distribution of ketose among the components of the tuber carbohydrate*

(Where the dates correspond these analyses refer to the extracts described in Table 1. The figures for individual components are in some cases the results of a single ketose estimation, but in others represent the mean of several, e.g. spots 1, 2 and 3 for 8 Feb. 1949 are each the mean of ten estimations.)

Date bought	Date dug up	Date extracted	TRS after hydrolysis (g./100 g. fresh tuber)	Ketose as percentage of TRS after hydrolysis	Ketose as % total ketose recovered from chromatogram					
					Fructose spot	Spot 1	Spot 2	Spot 3	Spot 4	Spot 5	Spot 6
8 Feb. 1949	—	8 Feb. 1949	12·3	79	—	10·8	10·5	11·7	10·9	10·8	—
—	16 Sept. 1949	16 Sept. 1949	—	—	—	2·2	2·5	2·5	—	—	—
—	28 Sept. 1949	6 Oct. 1949	14·4	91	1·5	3·7	3·0	2·8	2·8	3·0	—
—	28 Sept. 1949	17 Oct. 1949	13·3	86	4·2	5·4	3·3	4·4	4·1	4·6	—
—	28 Sept. 1949	24 Oct. 1949	14·2	90	2·2	7·2	5·3	6·6	3·8	4·3	4·9
—	28 Sept. 1949	31 Oct. 1949	15·3	82	2·6	6·3	3·9	5·0	5·3	5·8	—
—	28 Sept. 1949	7 Nov. 1949	16·0	86	1·3	6·6	4·2	6·3	5·5	5·9	—
—	28 Sept. 1949	24 Nov. 1949	19·4	85	—	7·7	5·0	6·5	6·6	6·7	—
—	27 Oct. 1949	23 Nov. 1949	—	—	—	7·3	7·3	8·4	8·0	—	—
—	24 Nov. 1949	24 Nov. 1949	14·4	73	—	9·4	8·7	10·7	9·9	—	—
24 Nov. 1949	—	24 Nov. 1949	18·9	83	—	4·9	4·7	6·6	6·3	6·2	—
24 Nov. 1949	—	4 Jan. 1950*	—	—	—	7·9	7·1	9·1	—	—	—
24 Nov. 1949	—	14 Feb. 1950*	—	82	1·3	8·8	9·3	10·5	—	—	—
24 Nov. 1949	—	3 Mar. 1950*	—	—	1·4	9·2	7·9	9·2	—	—	—
24 Nov. 1949	—	15 Mar. 1950*	—	—	2·1	9·9	8·8	8·8	—	—	—
—	5 Apr. 1950	1 May 1950	10·6	85	—	11·5	9·9	10·4	—	—	—
—	5 Apr. 1950	1 May 1950	10·9	83	—	11·5	10·1	10·6	—	—	—

* Tubers peeled before extraction.

present, occupied positions below sucrose, cf. Fig. 2. It is convenient to refer to the spot in the sucrose position as 'spot 1', to the one above it as 'spot 2' and so on, the spot with the lowest R_F being referred to as 'spot n'. There was very little monosaccharide, none of it pentose. Spot 1 had an R_F indistinguishable from that of sucrose in all solvent mixtures used. It gave the brown colour characteristic of free or combined glucose with benzidine-trichloroacetic. Using this reagent the colour of the higher spots was more and more yellow as the series was ascended to $R_F = 0$, suggesting a higher fructose to glucose ratio.

To test the homogeneity of the spots, two-dimensional chromatograms were run. Using butanol-acetic in both directions the spots were found to lie in a straight line, and there was no resolution of any spot into two or more components. This excludes the possibility that the R_F's of the spots are influenced by the presence of other members of the series. With butanol-acetic and phenol no evidence was found for heterogeneity; this is confirmed by the behaviour of fractions of higher R_F (mentioned below) originally separated by the use of phenol, which showed the same number of components whether run in that solvent or in butanol-acetic. These observations do not exclude the possibility that the spots represent mixtures of similar substances, particularly those of lower R_F, which must be assumed to consist of higher oligosaccharides. The spots occupy the same relative positions in phenol as in butanol-acetic.

Quantitative. The ketose content of the spots was estimated in a number of extracts (see Table 4).

The results demonstrate the even distribution of ketose among the components of higher R_F, independently of their contribution to the total ketose. The most noticeable deviation in this respect is spot 3, which seems always to have a higher ketose

content than spots 2 or 4. Estimations of ketose and of combined TRS in spots 1–3 were made as described under 'Methods' (see Table 5 for some typical results with one extract). It will be seen that the ratios of fructose to glucose in spots 2 and 3, calculated on the assumption that the substances are non-reducing, vary according to the basis of calculation. Small errors in the calculation of combined TRS have relatively large effects on the ratio, but an alternative explanation of the discrepancies may be found in slight hydrolysis during the drying process. On the other hand, the assumption that these spots are non-reducing may be incorrect; they may represent mixtures of reducing and non-reducing sugars.

action of purified preparations of invertase cannot be taken as evidence for the presence of sucrose in the extracts (cf. Colin, 1919).

Partial separation of the carbohydrates. A streak of 1·00 ml. of boiled extract, 45 cm. long, was applied 9·5 cm. from one edge of a sheet of filter paper (57 × 47 cm.). This was done by hand in four successive applications from a graduated pipette with a finely drawn tip, the applications being of 0·225, 0·33, 0·375 and 0·07 ml. Guide spots were placed at each end of the streak. The sheet was run for 92 hr. with phenol saturated with water, and the guide strips cut out and sprayed with orcinol. Because of the large quantity of material put on (101 mg. TRS)

Table 5. *TRS and ketose determinations on individual components of the tuber carbohydrate*

(An extract made on 14 Feb. 1950 from tubers bought 24 Nov. 1949, deproteinized with Pb, was used. 5 or 7 μl. spots were placed on chromatograms, and these were run together in groups *A*, *B* and *C*, all in butanol-acetic for 3 days. The extract was analysed using ordinary pipettes, and also using the 'Agla' syringe: 81·8 (ordinary), 81·1 (Agla) mg. TRS/ml. after hydrolysis; 67·2 (ordinary), 65·7 (Agla) mg. ketose/ml. All results are expressed in μg./50 μl. original extract; TRS is calculated as μg. hexose on basis of a fructose standard.)

Spot	Chromatograms	TRS before hydrolysis (p)	TRS after hydrolysis (q)	Ketose (r)	Glucose as 1·06 ($q-p-r$) (s)	TRS of blanks from same sheet corrected for area (t)	Glucose as 1·06 ($q-r-t$) (u)	Ratio fructose/ glucose from (s)	Ratio fructose/ glucose from (u)
1	*A*	—	602	270	—	76	271	—	1·0
	B	147	658	316	199	—	—	1·6	—
	C	178	668	260	245	166	258	1·1	1·0
2	*A*	—	494	282	—	67	156	—	1·8
	B	125	521	328	68	—	—	4·8	—
	C	158	542	284	106	124	141	2·7	2·0
3	*A*	—	489	322	—	56	118	—	2·7
	B	118	533	356	60	—	—	5·9	—
	C	113	532	315	111	104	121	2·8	2·6

Treatment with invertase. When two-dimensional chromatograms of tuber extracts were treated with invertase between the first and second developments, spots in the glucose and fructose positions were obtained from every component except the material with R_F approximately zero. In this case fructose appeared, but glucose could not be detected with certainty. This was not unexpected owing to the small proportion of glucose in this material (see Table 6). Faint spots also appeared with R_F values higher than that of the original spot from which they derived, but similar to original components with higher R_F. Thus, for example, spot 2 gave rise to a very faint spot in position 1, and spot 5 to spots in positions 4 and 3. Many such 'daughter spots' were slightly retarded in position when compared to original components with similar R_F, and, although it appears that invertase produces shorter-chain oligosaccharides from each component, further evidence is necessary to prove them identical with those occurring in the tuber extract. It is obvious that the appearance of reducing sugar through the only the spot in the sucrose position had separated cleanly and this occupied the region between 220 and 290 mm. from the point of application. The remaining streak was arbitrarily divided into four zones by cuts 9, 45, 155 and 220 mm. from the starting line. The corresponding areas from the unsprayed sheet were then cut out. The strips were extracted with water *in vacuo* at 40–45° in a Soxhlet extraction apparatus, each being extracted six to nine times. The volume of water was such that only about 1 ml. was left in the flask at the last evaporation. The final liquid remaining in the extraction chamber was tested for fructose by the Seliwanoff method with negative or only very slightly positive results, indicating a loss of less than 1 % of the total fructose at this stage of the recovery. The concentrated extracts, which were yellow in colour, were each made up to 5 ml. Chromatograms showed that fraction *A* (R_F corresponding to that of sucrose) was pure, that *B* contained two spots, and that *C*, *D* and *E* were probably more heterogeneous. An analysis of these fractions is given in Table 6.

Table 6. *Analysis of fractions separated chromatographically from tuber extract*

(For details of experiments see text, p. 120. Values calculated/ml. original extract.)

Fraction	TRS* after hydrolysis (mg.)	Ketose (mg.)	Glucose (by difference × 1·06) (mg.)	Glucose as % total monosaccharide after hydrolysis
A	15·8	7·7	8·6	52·5
B	20·75	13·0	8·2	38·7
C	28·15	22·35	6·15	21·6
D	23·25	21·0	2·4	10·3
E	10·9	10·8	0·1	(1)
Total recovered	98·85	74·85	25·45	—
Total in original extract	101	80·0	22·0	—
Recovery	98%	94%	—	—

* Calculated as hexose on basis of fructose standard.

Carbohydrates in other parts of the artichoke plant

Source of material. Plants raised from the tubers described above were used. Leaves were taken to the laboratory as quickly as possible in a cooled vacuum flask; the stems, which were long and heavy, were cut into short lengths.

Extraction and deproteinization. The stems and roots were extracted and the extract deproteinized by essentially the same procedures as were used for tubers. Leaves were extracted and deproteinized simultaneously in the Waring blender. A typical extraction was as follows: 50 g. of lamina were blended with 100 ml. of 35% (v/v) aqueous ethanol (containing potassium cyanide) and 35 ml. of chloroform, N-hydrochloric acid being added cautiously drop by drop to make the mash react green with bromocresol green. The whole mash was centrifuged and the upper layer (yellow with a slight tinge of green) was filtered with the addition of a little filter aid (Hyflo-Supercel, Johns-Manville Co. Ltd., London). Such extracts gave no precipitate with trichloroacetic acid. They were adjusted to pH 7 before concentration *in vacuo* with a bath temperature of 50° or less.

Roots. These showed the same series of components as the tubers when chromatographed.

Stems. The stem, particularly in the lower parts, contained a substantial pith, which could be cut out without much difficulty (Thaysen *et al.* 1929). Chromatographic examination showed a series of components similar to those of the tuber, both in extracts of the pith, and of the upper stem (see Fig. 2). As judged qualitatively there was more of the components of low R_F, and inulin separated from extracts kept in the refrigerator.

Inner and outer regions of immature tubers. The young tubers broke naturally into an outer layer and an inner core. There was no qualitative difference between them in respect of carbohydrate (Fig. 2).

Leaves. The amounts of fructose in the upper parts of the stem, and in the petioles and leaves, were lower than those in the other parts of the plant, so that concentration of the extracts was necessary as a preliminary to chromatographing them. The concentrated extracts failed to give satisfactory chromatograms, the whole of the carbohydrate remaining as a blob or streak, with few signs of differentiation. This effect (cf. Consden *et al.* 1944) could be reproduced artificially to some extent: 7 μl. spots of M-potassium dihydrogen phosphate solution were placed on the starting line of a strip of paper, and after they had dried, 5 μl. spots of a control mixture of sugars and of a tuber extract known to give satisfactory chromatograms were applied at the same point. When run in butanol-acetic the chromatograms showed retardation and distortion of the spots, though not as pronounced as that seen with the leaf extracts. Application of 7 μl. spots of 30% (w/v) lead acetate ($Pb(C_2H_3O_2)_2.3H_2O$) and 40% (w/v) barium acetate ($Ba(C_2H_3O_2)_2.H_2O$), which it was thought might precipitate interfering substances, had no effect on the R_F's of the control mixture of sugars, nor of the tuber extract. It would thus seem that it is not salt concentration alone that is responsible for the failure to achieve satisfactory separation (cf. Partridge, 1948).

Attempts to remove salts from the leaf extracts by the use of ion-exchange resins (in which we were greatly helped by Dr R. E. Kressman and the Permutit Company) had only limited success in the summer of 1949. The cations were removed readily by Zeo-Karb 217, but the resulting acid solution was brought towards neutrality only slowly, even by the more basic resins of the 'Deacidite' series (up to '*F*'). The treated solutions, which had been cycled repeatedly through columns of the two types of resin gave improved, but not entirely satisfactory, chromatograms, in which 'spot 1', glucose and fructose were always seen. In one or two extracts indication was given of material with lower R_F than

sucrose, but in none was there evidence of a series of components resembling the tuber extract. Starch is present in the leaves (Brown & Morris, 1893).

family (cf. Willis, 1931) were represented (see Table 7).

Underground organs (100 g.) (after removal of the fibrous roots) were disintegrated in the Waring blender with an equal weight of 0·01 M-potassium cyanide for about 5 min. In most cases some difficulty was experienced in the blending because of the fibrous or woody nature of the material. The most notable exception (except for the dahlia tubers

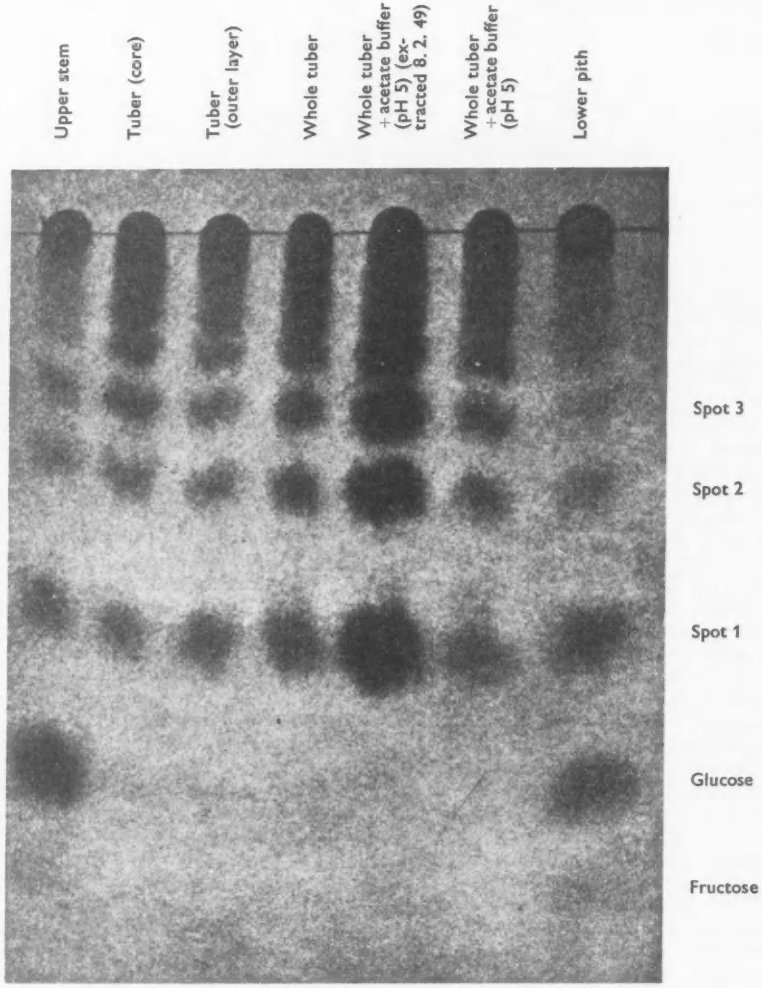


Fig. 2. Chromatograms of extracts of various parts of artichoke tubers and stems. Run in butanol-acetic for 40 hr.; sprayed with benzidine-trichloroacetic acid. Extracts made from plant 16 Sept. 1949 unless otherwise indicated (see Fig. 3). (First positive print.)

Carbohydrates in other members of the Compositae

The storage organs of other arbitrarily selected species of the Compositae were investigated chromatographically. Members of both subgroups of the

Table 7. *Details of plants examined*

(All from the Sheffield area.)

Subfamily	Systematic name	Common name	Part of plant examined	Date of collection (1949)
Tubuliflorae	*Dahlia* sp.	Garden dahlia 'Baby Rose'	Tuber	1 Sept.
	Tussilago farfara L.	Coltsfoot	Rootstock	15 June
	Helianthus tuberosus L.	Jerusalem artichoke	All vegetative organs	—
Liguliflorae	*Lactuca sativa* L.	Lettuce, 'Tom Thumb'	Taproot	16 August
	Leontodon autumnalis L.	Autumnal hawkbit	Rootstock	20 July 26 August
	Sonchus oleraceus L.	Common sowthistle	Rootstock	20 July
	Taraxacum officinale Weber	Dandelion	Rootstock	27 April
	Tragopogon pratensis L.	Goatsbeard	Taproot	4 July

which resembled artichoke tubers in their ease of handling) was *Leontodon autumnalis* which had crisp, parenchymatous rootstocks with little fibrous material. The extracts obtained after squeezing the material through gauze were light-brown or yellow in colour, of pH about 6·0–6·5 and more or less opaque. They were heated in a boiling-water bath for 15 min. as soon as possible after preparation. These boiled extracts were concentrated *in vacuo* to a thick syrup and taken up in water to give sufficient concentration of carbohydrate to allow of chromatographic investigation, namely 5–10% (w/v) total fructose. When extracts so concentrated were kept in the refrigerator there was precipitation (to a lesser or greater degree) of material, presumably inulin, soluble in hot but not in cold water. The amount of this precipitate was small, except in one case. The whole concentrated extract of *Leontodon autumnalis*, however, set solid, even at room temperature, and a relatively large quantity of polysaccharide was isolated from it, the yield from 70 ml. of original unconcentrated extract being 0·7 g. In spite of considerable salt effects (especially in the case of *Sonchus oleraceus*), it was obvious that a series of carbohydrates comparable to that in artichoke tuber extracts was present in every case (see Fig. 3).

DISCUSSION

The carbohydrates of the artichoke tuber

These results demonstrate the heterogeneity of the tuber carbohydrates; they also provide a basis for the separation and estimation of the components of higher R_F. It is to be noted that the latter constitute a considerable proportion of the total carbohydrate: the five spots with R_F's ranging from that of sucrose to about 0·025 (in butanol-acetic) contain more than half the fructose of some extracts, and if their higher relative content of glucose is assumed from the qualitative and quantitative data available they would comprise an even higher proportion of the total sugar. The even distribution of fructose among these components in all the extracts examined would seem to contradict the claims of Tanret (1893*b*) that his 'helianthénine' and 'synanthrine' were chemical entities, since in his analysis these two fractions and sucrose occurred in a ratio of approximately 1 : 8 : 2, the three constituting two-thirds of the total carbohydrate of tubers stored until June.

It is evident that considerable possibilities exist for variation in over-all composition through variations in the relative proportions of the many components. In practice this would seem to be the explanation for the variations in fructose, as percentage of total carbohydrate, and it seems likely that the optical rotation of the mixture will depend on similar variations, the components with higher R_F probably having more positive specific rotations. It is not clear, however, whether any considerable change could occur within a given mixture of inulin, inulides and sucrose without an accompanying change either in the ratio of fructose to glucose, or in the proportion of free hexose present.

It is difficult to avoid the assumption that all the carbohydrates present in the tuber bear a relation to inulin; that they represent a series terminated by inulin at one end, and bounded by sucrose at the other, all members having the typical 1:2-linked fructofuranoside grouping and bearing a glucopyranoside as a non-reducing end group (I). Hirst, McGilvray & Percival (1950) have reported the isolation of 2·2% of tetramethyl glucopyranoside from methylated dahlia inulin, and have postulated a sucrose-like terminal group for the molecule. In addition they found circumstantial evidence for 2·6% of 2:4:6-trimethylglucose, which would indicate the presence of glucose within the chain (cf. Irvine & Montgomery, 1933). The total of methylated glucose agrees well with the percentage of glucose estimated by their chromatographic procedure, i.e. 5·7%, but is considerably higher than the figures given by others (cf. McDonald, 1946; Bell & Palmer, 1949) which lie between 1·5 and 2·5%. We have not been able to trace the source of the attribution by Lemoigne (1941*a*) to Kiliani of a formula for inulin, $(C_6H_{10}O_5)_4C_{12}H_{22}O_{11}$, in which the group $C_{12}H_{22}O_{11}$ is intended to represent sucrose.

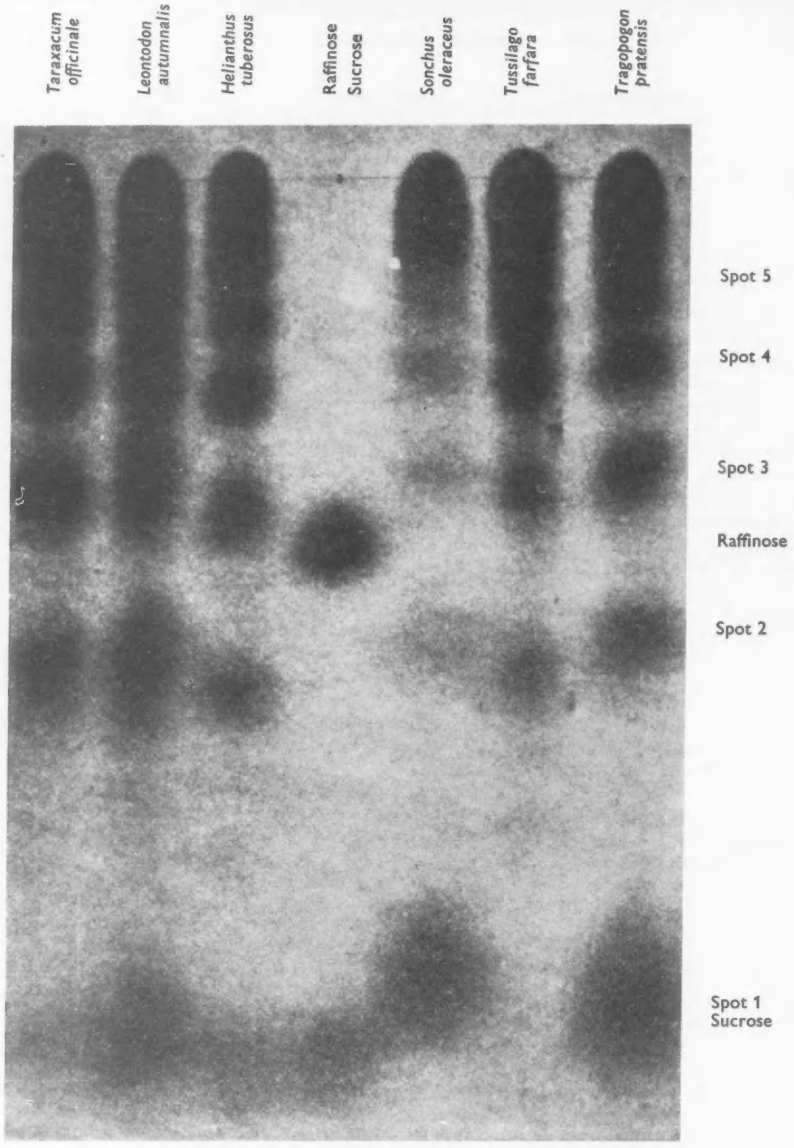
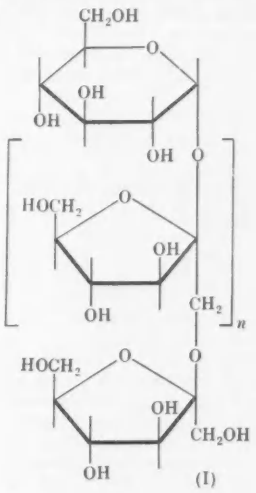


Fig. 3. Chromatograms of extracts of subterranean organs of six species of Compositae. Run in butanol-acetic for 6 days; sprayed with phloroglucinol. (First positive print.)

Attempts to support this hypothesis by analysis of spots 2, 3 and 4 in the paper chromatogram have been vitiated by certain difficulties in the estimation.



(I)

The carbohydrates in other parts of the artichoke plant

Although no extensive investigation has been undertaken (no flowers or seeds were available), it is evident that inulin and inulides are present in all organs of the artichoke plant, with the possible exception of the leaves; this confirms the observations of Colin (1919). In some instances the effect of interfering substances in the paper chromatogram distorted or delayed the movement of the carbohydrates, and until this difficulty has been overcome a satisfactory quantitative study of the whole plant will not be possible. Until then it must remain an open question, for example, whether inulin is in fact made in the tubers or merely translocated to them, although in view of the homology of stem and tuber it would seem likely that the polysaccharide may be synthesized in both parts.

The carbohydrates of Compositae

The general similarity of the chromatograms made with extracts of other Compositae makes it probable that any conclusions drawn from an investigation of the artichoke tuber may be applied tentatively to all members of this family, and that in this way general information may be gained about the synthesis and breakdown of inulin.

SUMMARY

1. The carbohydrates of the Jerusalem artichoke (*Helianthus tuberosus* L.) and of several other members of the Compositae have been examined by various techniques including the use of qualitative and quantitative paper partition chromatography.

2. The existence has been demonstrated of a series of at least seven non-reducing substances with R_F's ranging from that of sucrose to zero, consisting chiefly of fructofuranoside residues, but containing glucose residues in proportions decreasing with R_F.

3. These substances are present in the stems, tubers and roots of the Jerusalem artichoke, and in the underground organs of seven other species of Compositae.

4. In all extracts examined fructose residues were distributed evenly among those components with R_F's sufficiently high for them to be isolated for analysis.

5. In the artichoke tuber the proportion of the total fructose residues contributed by the five components with greatest R_F's varies from 15% in September to 55% in early spring.

6. Some of the implications of these findings are discussed.

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REFERENCES

Bacon, J. S. D. & Bell, D. J. (1948). *Biochem. J.* **42**, 397.
Bacon, J. S. D. & Edelman, J. (1949). *Biochem. J.* **45**, xxviii.
Bell, D. J. & Palmer, A. (1949). *Biochem. J.* **45**, xiv.
Brown, H. T. & Morris, G. H. (1893). *J. chem. Soc.* p. 604.
Colin, H. (1919). *Rev. gén. Bot.* **31**, 70, 179, 229, 277.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.
Edelman, J. & Bacon, J. S. D. (1949). *Biochem. J.* **45**, xxix.
Edelman, J. & Bacon, J. S. D. (1950). (Unpublished communication.)
Folin, O. (1929). *Laboratory Manual of Biological Chemistry*, 4th ed. p. 99. New York and London: Appleton.
Green, J. R. (1888). *Ann. Bot. Lond.* **1**, 223.
Hirst, E. L., McGilvray, D. I. & Percival, E. G. V. (1950). *J. chem. Soc.* p. 1297.
Horrocks, R. H. & Manning, G. B. (1949). *Lancet*, **1**, 1042.
Irvine, J. C. & Montgomery, T. N. (1933). *J. Amer. chem. Soc.* **55**, 1988.
Jackson, R. F. & Goergen, S. M. (1929). *Bur. Stand. J. Res., Wash.*, **3**, 27.
Keilin, D. & Hartree, E. F. (1948). *Biochem. J.* **42**, 230.
Lemoigne, M. (1941*a*). *Ann. Ferment.* **6**, 193, 257.
Lemoigne, M. (1941*b*). *Ann. Ferment.* **6**, 201.
Lemoigne, M. (1941*c*). *Ann. Ferment.* **6**, 273.

Lemoigne, M. (1942). *Ann. Ferment.* **7**, 1, 129.
McDonald, E. J. (1946). *Advances in Carbohydrate Chemistry*, **2**, 253. New York: Academic Press.
Miller, B. F. & Van Slyke, D. D. (1936). *J. biol. Chem.* **114**, 583.
Patridge, S. M. (1948). *Biochem. J.* **42**, 238.
Partridge, S. M. (1949). *Nature, Lond.*, **164**, 443.
Rose, V. (1804). *Gehlens Neues allgemeines Journal der Chemie*, **3**, 217.
Tanret, C. (1893*a*). *Bull. Soc. chim., Paris*, **9**, 200.
Tanret, C. (1893*b*). *Bull. Soc. chim., Paris*, **9**, 622.
Thaysen, A. C., Bakes, W. E. & Green, B. M. (1929). *Biochem. J.* **23**, 444.
Willis, J. C. (1931). *Flowering Plants and Ferns*, 6th ed. Cambridge: University Press.
Wolff, J. (1916). *C.R. Acad. Sci., Paris*, **162**, 514.
Wolff, J. & Geslin, B. (1918). *Ann. Inst. Pasteur*, **32**, 71.

The Separation of *N*-2:4-Dinitrophenyl Amino-Acids on Paper Chromatograms

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The method of identification and estimation of the free amino-groups of proteins, first applied to insulin by Sanger (1945), has since been widely used. The terminal amino-acids of the peptide chains which carry free amino groups are isolated as their *N*-2:4-dinitrophenyl (DNP) derivatives, which can be separated on silica gel partition chromatograms (Sanger, 1945). Buffered columns may also be employed with a variety of solvent systems (Blackburn, 1949).

A number of attempts to separate DNP-amino-acids on paper chromatograms have met with only limited success due to 'tailing' of the spots (Sanger, 1945; Blackburn, 1949). Phillips & Stephen (1948) separated some of the DNP-amino-acids, particularly the slower moving ones, using a two-dimensional method, but also observed marked 'tailing' in many solvents. In contrast they found that fairly large DNP peptides formed good spots on paper chromatograms. Woolley (1949) also found that some DNP peptides from enzymic digests of DNP-insulin ran as single spots on phenol-ammonia or butanol-ammonia chromatograms.

We have now observed that DNP-amino-acids may be successfully separated on buffered one-dimensional paper chromatograms, compact spots without 'tailing' and possessing characteristic rates (R_F values) being formed in suitable solvents. After the present paper had been read to the Biochemical Society (Blackburn & Lowther, 1950) we received from Prof. C. Fromageot a copy of a paper by Monier & Pénasse (1950), who separated mixtures of DNP-amino-acids on paper using solvents saturated with potassium benzoate solution.

EXPERIMENTAL

The chromatograms were run on strips, approx. 12 × 35 cm., of Whatman no. 4 filter paper which had been previously soaked in buffer solution and dried at room temperature. Phthalate buffer of pH 6·0 (Britton, 1942, Table 70) proved generally suitable, and was used except where otherwise stated. About 2–5 μg. of a DNP-amino-acid (dissolved in a suitable solvent) was applied to the paper. The papers were then fixed in small stainless steel troughs mounted in glass battery jars, water and solvent being placed in the bottom of the jar, and the top covered with a sheet of plate glass. The closed box was allowed to stand for some time before the solvent (previously saturated at room temperature with the same buffer that was applied to the paper) was introduced into the trough. The movement of the DNP-amino-acids down the paper could be observed during the course of the experiment. Adequate separations were generally obtained in a run lasting several hours, or occasionally overnight.

Solvent systems

Many of the solvent systems tried proved unsuitable, because the DNP-amino-acids ran fast near the solvent front or else 'tailed' somewhat. The solvent systems of general use are described below.

tert.-*Amyl alcohol.* All the DNP-amino-acids move as well defined spots in this solvent.

Propanol-cyclo*hexane.* A mixture of DNP-leucine, DNP-valine, DNP-alanine and DNP-glycine separates into four clearly defined spots in 30% propanol-*cyclo*hexane (Fig. 1). Occasionally difficulty may be experienced with this solvent, owing to the volatility of *cyclo*hexane, and 'tailing' spots result. This may be overcome by substituting light petroleum (b.p. 100–120°) for the *cyclo*hexane, when similar separations can be achieved.

n-*Butanol and benzyl alcohol.* The spots formed in these solvents are slightly elongated, but the addition of 10% of ethanol (v/v) before saturation with buffer causes more compact spots to be formed. 10% ethanol-benzyl alcohol is superior to 10% ethanol-butanol, as the differences in R_F values between the different DNP-amino-acids are greater.

Ethyl acetate and 1% *butanol-chloroform* are not as generally useful as the above solvents. Ethyl acetate may be used for the separation of the slower moving DNP-amino-acids on pH 8 phosphate-citrate buffer paper (Britton, 1942, Table 65). DNP-threonine and DNP-serine can be separated from each other and from DNP-glutamic acid and DNP-aspartic acid, both of which move slowly. The DNP-mono-

amino-acids, however, give rather elongated spots in this solvent. DNP-threonine, DNP-serine, DNP-glutamic acid and DNP-aspartic acid do not move in 1% butanol-$CHCl_3$ while the DNP-monoamino-acids move fairly fast down the paper. DNP-glycine and DNP-alanine, however, may be separated from each other.

the same reason, but Table 1 gives some values for a few representative solvents.

It should be possible to perform end-group estimations on a micro scale by comparing the size and intensity of the DNP-amino-acid spots with those given by known amounts of DNP-amino-acids run simultaneously on the same chromatogram. After drying, photographic copies of the chromatograms may be made on contact paper, when the spots show up clearly. A hydrolysate of DNP-insulin, when examined in 30% propanol-*cyclo*hexane, gave two spots in a similar position to those given by a mixture of DNP-phenylalanine and DNP-glycine. Phenylalanine and glycine are known to be the terminal amino-acid residues of insulin (Sanger, 1945).

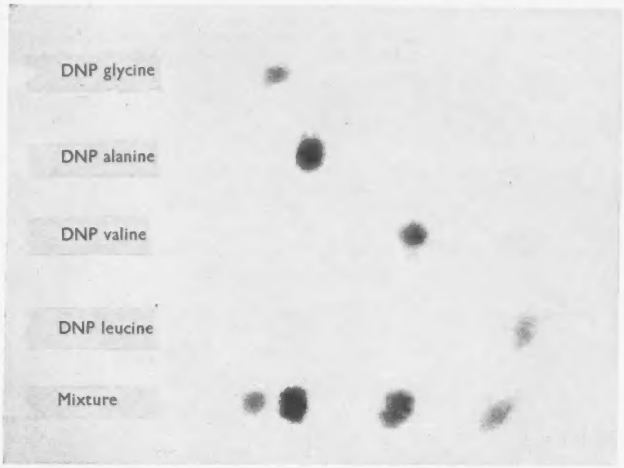

Fig. 1. Chromatogram of DNP-amino-acids run in 30% propanol-*cyclo*hexane.

The R_F values of the individual DNP-amino-acids vary with the pH of the buffer applied to the paper, but slight variations do not prevent the necessary separations. The R_F values also appear to vary with the temperature, probably due to alteration in the composition of the solvent mixtures.

Table 1. *The R_F values of DNP-amino-acids on buffered paper*

	Solvent system		
	30% Propanol-*cyclo*hexane	Tertiary amyl alcohol	10% Ethanol-benzyl alcohol
DNP-leucine	0·28	0·88	0·71
DNP-valine	0·23	0·79	0·59
DNP-phenylalanine	0·22	0·74	0·63
DNP-alanine	0·15	0·46	0·36
DNP-glycine	0·10	0·23	0·26
DNP-threonine	0·07	0·36	0·26
DNP-serine	0·05	0·21	0·18
DNP-glutamic acid	0·05	0·04	0·07
DNP-aspartic acid	0·02	Slow	0·03

For these reasons, identification of a DNP-amino-acid is never based on the R_F values alone, control DNP-amino-acids always being run on the same sheet of paper as the unknown. Extensive Tables of R_F values are not quoted for

DISCUSSION

The DNP-amino-acids run most successfully on paper in solvents where the mobile phase has a high water content. This high water content may prevent association of the DNP-amino-acids, which can lead to 'tailing'. The phthalic acid of the buffer, which is slightly soluble in the mobile phase, probably also helps in preventing this association (cf. Baker, Dobson & Martin, 1948). When the amount of water in the mobile phase of a two-component system such as benzyl alcohol-water is increased by the addition of a third component, such as ethanol which is soluble in both phases, the DNP-amino-acid spots become much more compact in shape. If the water content of the mobile phase is too high, the R_F values are large and the difference between the values for different DNP-amino-acids is not sufficient to effect

separations. This occurs with *sec.*-butanol saturated with pH 6 buffer.

When DNP-amino-acids are run on paper with water or buffer as solvent, they travel down the paper as 'spots' some distance behind the solvent front. With methanol or ethanol on pH 6 buffered paper, fairly well-defined 'spots' with some 'tailing' are formed, the separation between individual DNP-amino-acids being greater than in water alone. When non-hydroxylic organic solvents not containing water are similarly used, 'tailing' spots are formed.

SUMMARY

A method for the separation of *N*-2:4-dinitrophenyl amino-acids on buffered paper chromatograms is described.

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REFERENCES

Baker, P. B., Dobson, F. & Martin, A. J. P. (1948). (Unpublished results.) Cited by Martin, A. J. P. (1949). *Biochem. Soc. Symp.* **3**, 4.

Blackburn, S. (1949). *Biochem. J.* **45**, 579.

Blackburn, S. & Lowther, A. G. (1950). *Biochem. J.* **46**, xxvii.

Britton, H. T. S. (1942). *Hydrogen Ions*, **1**, 306 (Table 70), 304 (Table 65). London: Chapman and Hall.

Monier, R. & Pénasse, L. (1950). Paper read to Academy of Sciences, Paris, 20 March 1950.

Phillips, D. M. P. & Stephen, J. M. L. (1948). *Nature, Lond.*, **162**, 152.

Sanger, F. (1945). *Biochem. J.* **39**, 507.

Woolley, D. W. (1949). *J. biol. Chem.* **179**, 593.